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EFFECT OF LYMPHOKINES AND GROWTH FACTORS ON COLONY FORMATION AND LEUKEMIC CELL DIFFERENTIATION

BY

© SIMEON VASSILIADIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

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Date: Sept. 3, 1985

TO MY DAUGHTER MARINA - ANNA

AND

MY WIFE IRENE

ABSTRACT

We have investigated in this study the growth patterns of a panel of cell lines towards a battery of factors. The observations were focused on HL-60, a promyelocytic leukemia cell line, while other cell lines were used for comparative purposes.

A model-system was developed in which the effect of factors on proliferation by leukemic and normal cells could be assessed. This system used Human Bone Marrow Layers as a supportive matrix.

In addition, we were able to induce expression of class II and monocytic markers by gamma Interferon and various conditioned media respectively. However, gamma Interferon always suppressed differentiation as assessed by colony formation.

Using an arsenal of factors in order to reverse the suppressive effect of this agent, we discovered that Interleukin 2 acted in an antagonistic manner to gamma Interferon.

We were also able to detect, for the first time, an increase of the c-myc's (a cellular oncogene) mRNA during long term treatment with gamma Interferon.

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CHAPTER I

INTRODUCTION

The purpose of the present work is to seek information on the reciprocal processes of cell proliferation and cell differentiation in the context of human hemopoiesis.

The field of hematology has been studied since the nineteenth century but many questions still remain unanswered. A complete investigation would be very extensive due to the multiplicity of the factors involved. For example, the bone marrow, unique source of blood cells, can be examined from several different viewpoints. This hemopoietic tissue is not only a reservoir of stem cells but is also actively involved in the control of cell proliferation and cell differentiation leading to the formation of all types of hemopoietic cells.

In this study, I have developed some in vitro models of hemopoiesis by using, on the one hand, a panel of leukemic cell lines representing different stages of hemopoietic differentiation and, on the other hand, Human Long Term Bone Marrow Cultures (HLTBMC).

A) HEMOPOIESIS SEEN AS A CELL PROLIFERATION-DIFFERENTIATION PROCESS

1) THE MARROW AS A HEMOPOIETIC TISSUE.

The marrow is one of the largest organs in the body. In normal adults, the net daily production is about 2.5 billion erythrocytes, 2.5 billion platelets and 0.1 billion granulocytes per kilogram of body weight (132). It is also the main producer of monocytes and lymphocytes.

Actually, the productive capacity of the marrow is dependent on the organism's needs and requirements. The different cell types appear to be mixed in a random way and to have no architectural positioning with regard to one another. In fact, there are speculations suggesting that there may be a degree of direct cell to cell inductive regulatory process(es) as described for other hemopoietic organs in mice (85).

The bone marrow has been best described by Metcalf and Moore (85). They suggest that its hemopoietic function depends, to a certain extent, on the microanatomy of the vascular network, and that the arterial supply to the marrow in connection to venous throttling arrangements between terminal sinuses in the marrow and periosteal veins indicate the existence of

blood flow-controlling mechanism(s) which may regulate hemopoietic activity and cell release. The latter, in turn suggests a degree of cell maturation achieved within the organ.

2) THE CONCEPT OF STEM CELL(S).

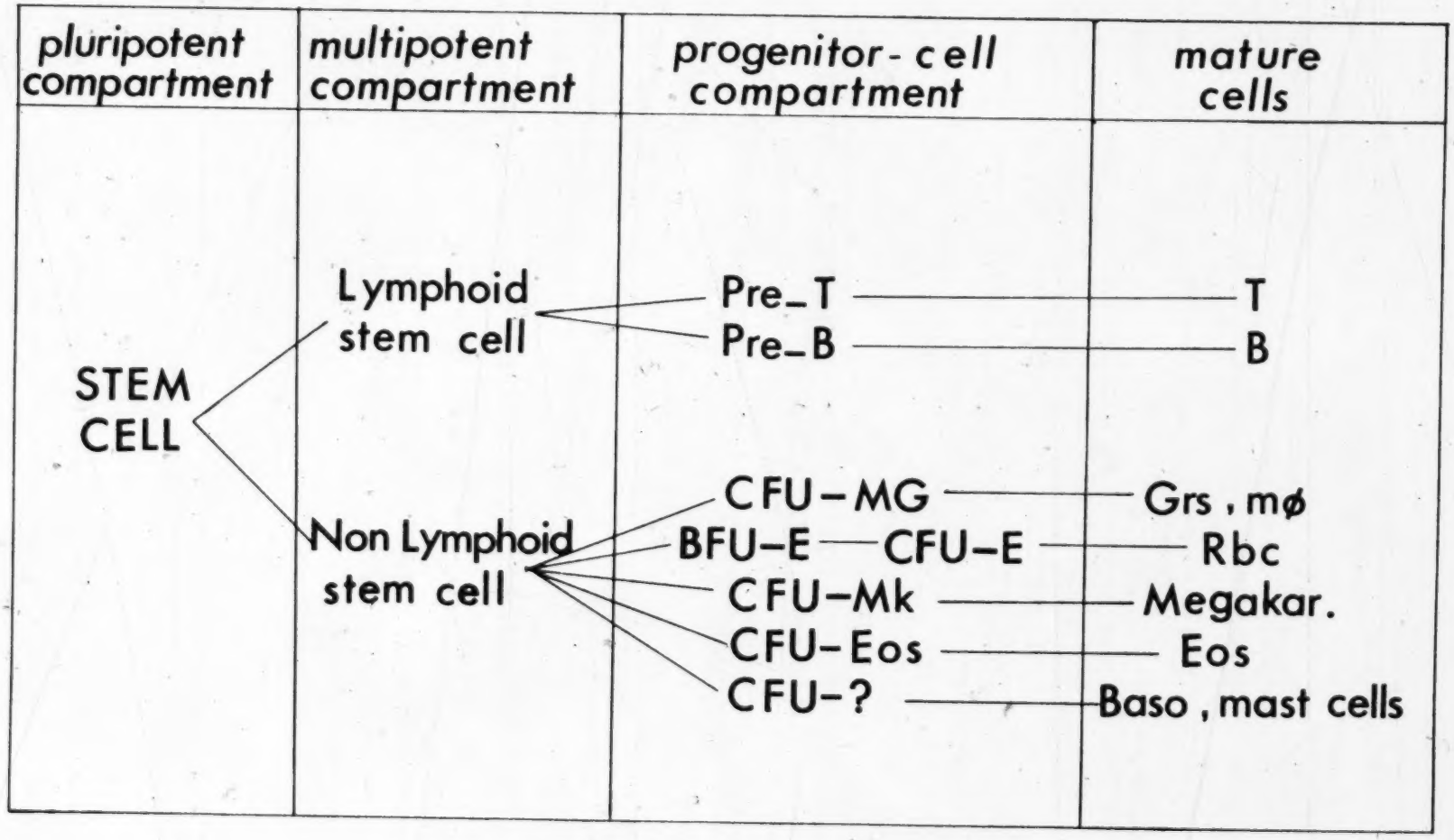
The stem cells consist of a pool of cells similar in morphology but dissimilar in function. Stem cells are capable of differentiation and self-renewal. The first observations relevant to determining the characteristics of stem cells came in 1961 when Till and McCulloch studied the formation of colonies in the spleen of bone marrow-reconstituted irradiated mice (121). These investigators showed that a single progenitor cell is capable of giving rise to a colony of cells, all of the same lineage. This study confirmed Maximow's theory (77), who in 1924 proposed the existence of one cell able to give rise to all blood cells, and that this cell could give rise to stem cells with separate pluripotentiality for the lymphoid and myeloid systems. He further envisaged that these cells could in turn produce stem cells of more restricted potentiality.

3) THE MARROW AS A RESERVOIR OF CELLS.

The earliest marrow stem cell is a pluripotential/totipotential cell capable of extensive, possibly lifelong, self-renewal and differentiation to hemopoietic and lymphocytic progenitor cells. The next cells in the hierarchy are the multipotential stem cells (hemopoietic or lymphopoietic cells capable of self-renewal and differentiation to unipotential hemopoietic or lymphopoietic progenitor cells). These progenitors support and provide cellular backup for stem cell pools committed to erythroid, megakaryocytic or granulocytic lineages. (An outline of the marrow cell compartments is given in Fig. 1). Between the pluripotential and the precursor cells more than one class of cells may be found. According to Boggs (7), in the neutrophil-monocyte pathway three such classes exist: the cell growing in diffusion chambers (CFU-D), the cell forming colonies in semisolid media (CFU-NM) and the cell forming clusters in the same semisolid media. In the erythroid line progenitor cells form large colonies or bursts (CFU-E/BFU-E), or form clusters.

It is not surprising that the frequency of stem

Fig. 1
Events taking place during normal hemopoiesis.



and progenitor cells is relatively low, i.e. between one in a thousand and one in ten thousand cells. This frequency can be influenced by the local microenvironment or regulatory factors that drive the stem cells to develop into a more differentiated state (83).

4) THE CONCEPT OF COMPETENCE

Self-renewal and commitment have been extensively studied by means of biological, biochemical and even mathematical approaches. Some investigators (122,53) proposed a stochastic model, which states that the decision by a stem cell to renew itself or to generate committed progenitors is governed by a distributional parameter p, which represents the probability of self-renewal of stem cells, and that many factors may have an influence on this parameter. An extension of this concept suggests that stem cell commitment is under the progressive and stochastic restriction of the differentiation potential of the hemopoietic stem cells. Other models, called deterministic, have been developed and are based upon the presence of regulatory mechanism(s) that could drive stem cells into a restricted pathway, i.e. either to self-renewal or to

differentiation. One important factor is the so called hematopoietic inductive microenvironment (HIM) which exercises its influence on the multipotential stem cells (123). Other suggestions lay stress on the importance of growth factors and other agents.

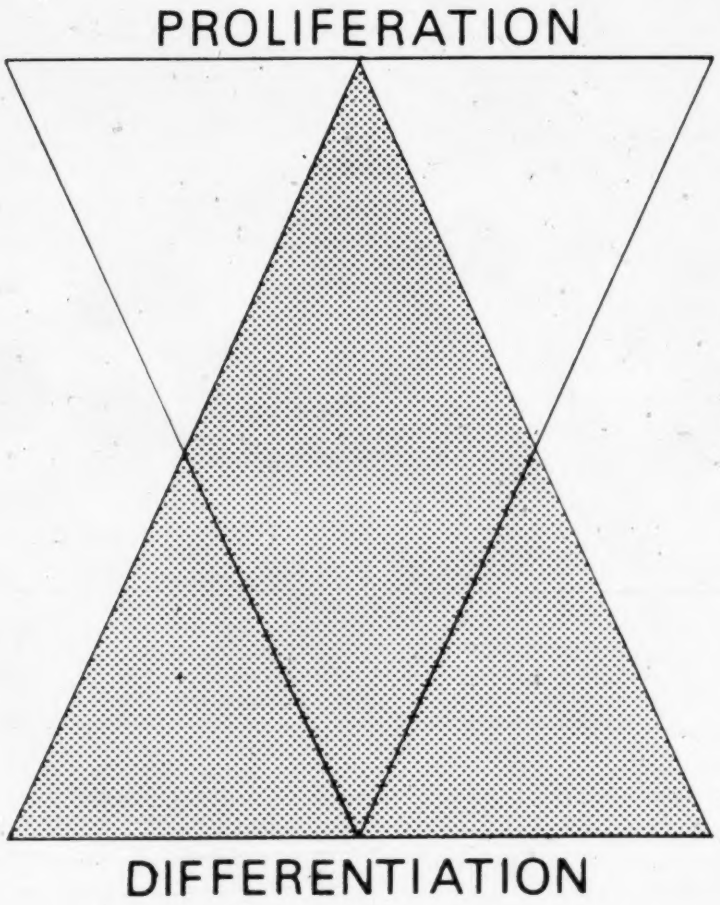
5) THE CONCEPT OF HEMOPOIESIS.

The pattern of normal hemopoiesis as illustrated in Fig.1 involves an equilibrium between cell proliferation and cell differentiation necessary to maintain the balance between the different hemopoietic cell compartments. The relationship between the two processes can be viewed by using the concept of Pascal's triangles (Fig. 2). Classically, differentiation has been seen as a cascade of events occuring through a succession of cell divisions (Fig. 1). However, it is known that the process of cell differentiation induces a negative feedback on proliferation (122).

All the above events are subject to dinstict and specific regulatory processes. These driving forces are not yet fully understood but their existence is demonstrated by in vitro and in vivo experimentation

Fig. 2
Relationship between proliferation and differentiation.

Pascal's triangles illustrate the intrarelationship between cell differentiation and cell proliferation.



in mice, identification of specific factors (CSFs, hemopoietins) as well as by the study of hematological diseases such as aplasia or myelo-proliferative disorders.

Observations on these diseases have led investigators to different conclusions, all supporting the concepts of pluripotential stem cells, lineage differentiation and existence of clonal mechanisms. For example, Chronic Myeloid Leukemia (CML) is clearly identified as a proliferative disorder occuring at the stem cell level but still permitting an almost complete differentiation to occur. CML is a clonal hemopathy occuring at a multipotential stem cell level as demonstrated by two genetic markers: the occurence of the Philadelphia chromosome in different lineages and the glucose-6-phosphate dehydrogenase (G6PD) allotype (80). For the former, the chromosome 22 known as the Philadelphia chromosome is characterized by a loss of its long arm, which in most CML patients is translocated to chromosome 9 (133). For the latter, in G6PD heterozygotes, the neoplastic clone is detected to be either type A or type B and only residual normal cell populations will have both enzyme types. Therefore, it is possible, using the G6PD system, to analyze not only the clonality of the neoplasm, but

also to determine stem cell lineage relationships. For example, in a G6PD heterozygote with CML, when circulating blood cells are tested, the red cells, platelets, eosinophils, monocytes and granulocytes all have a single enzyme type; whereas skin manifests both A and B types (36).

After blast transformation some cells show characteristics of granulocytic differentiation and in some instances exhibit erythroid, monocytic or megakaryocytic characters (42). When blast transformation occurs at another stage (it is difficult to assess if it is earlier or later) and involves uncommitted cells, CML resembles acute lymphocytic leukemia (ALL). Patients frequently exhibit elevated levels of terminal deoxynucleotidyl transferase (TdT), cytoplasmic immunoglobulin and the common acute lymphocytic leukemia antigen (CALLA) (133).

Acute Myeloid Leukemia (AML), which also occurs at a stem cell level, does not allow the progeny to differentiate until the late stages. Still a mature cell can be the malignant stem cell for the hemopathy. These are the so called "frozen stages" of differentiation. AML thus appears as a very active proliferative disorder in which proliferation seems to

overcome any possibility of differentiation. However, it is difficult to establish a border between the two types of leukemia, since AML cells could express or be induced to same differentiation stages, and the obligatory outcome of CML is the transformation into an acute leukemic-like disease (blast crisis) (5).

6) THE STUDY OF HEMOPOIESIS

Much of what is known about blood and blood formation is the result of elaborate studies on blood cell morphology. The present day notion that the cells which are found in the peripheral blood are the end products of a differentiation process which initiates in the bone marrow is derived from microscopical studies performed by pathologists at the turn of the century. But microscopy was not enough for the study of earlier stages of blood cells. Thus, during the last three decades, several techniques for the experimental manipulation of hemopoietic cells in vivo and in vitro have been developed. However, many questions remain unanswered and need further research.

In this study, simple assays were used for determining proliferation, differentiation, surface marker and cellular oncogene expression of leukemic

cells.

Proliferation refers to cell division but sometimes implies cell division plus differentiation and maturation. Except the microscopic assessement of proliferation, the most common way is by ^{3}H thymidine (^{3}H TdR) incorporation into actively dividing cells (9,10). Another way for assessing proliferation is in vivo repopulation which involves colony formation in the spleen (121).

Differentiation is the process whereby a dividing cell gives rise to progeny that differ from it qualitatively in terms of morphology, aquisition of specific functions (T cell populations) and elaboration of specific products (Immunoglobulins, hemoglobin, enzymes, e.t.c.). Differentiation can be thus detected by staining techniques, functional assays or bioassays.

In this work we used mainly colony assays on semisolid media in order to identify the progeny and to assess the efficiency of progenitor cells to form colonies. Hemopoietic cells are capable of giving colonies in vitro when stimulated by specific factors. Their formation is dependent on the addition of exogenous stimulators. The factors which are an essential requirement for in vitro colony formation have been

designated as Colony Stimulating Factors (CSF) (82,97,98). Their activity is found in homogenates of most tissues and they probably act in conjuction with a proper growth environment provided by an agar or methylcellulose matrix.

Our studies were mainly focused on the promyelocytic cell line HL-60 while other myeloid and erythroid cell lines were used as controls. HL-60 has been mostly studied in terms of proliferation and differentiation by using batteries of chemical compounds. We decided to begin our investigation with biological substances like lymphokines and growth factors. We expanded the research already done with gamma interferon (gamma IFN) and added alpha and beta IFNs plus interleukin-2 (IL-2). On the other hand, several CSFs were used whose effect was not thoroughly investigated by other teams. The growth factors we used are given in detail in the Methods chapter.

Surface marker expression was assessed by indirect immunofluorescent testing and cellular oncogene expression was approached by biochemical techniques.

All four different but intrarelated functions studied gave us insight of leukemic cell responsiveness during activation which may have some therapeutic value in the future.

B) LEUKEMIC CELL LINES

Cell lines derived from patients' peripheral blood, bone marrow or other tissues can be used to study pertinent aspects of cell proliferation and differentiation. These cell lines can respond to some differentiation factors like TPA, IFNs, CSFs, Retinoic Acid e.t.c. The lines can be maintained in culture for prolonged periods of time. They can be easily frozen and stored at -196^{o}C and used at any time afterwards. Thus, the cells are readily available in huge numbers and represent a quite homogeneous population. The analysis of events related to hemopoiesis at the genetic and biochemical levels is thus feasible, since in vitro survival can be sustained. The cell lines can be classified according to their morphology, their capability to synthesize specific proteins or enzymes and their terminal differentiation along the myeloid pathway (KG-1, ML1, ML2, ML3, EM1, EM2, HL-60), erythroid pathway (HEL), monocytic (U-937), or to some undifferentiated state (K 562). The relationship between the cell lines and their main characteristics are given in Fig.3 and Table 1 respectively.

Fig. 3
Relationship between leukemic cell lines.

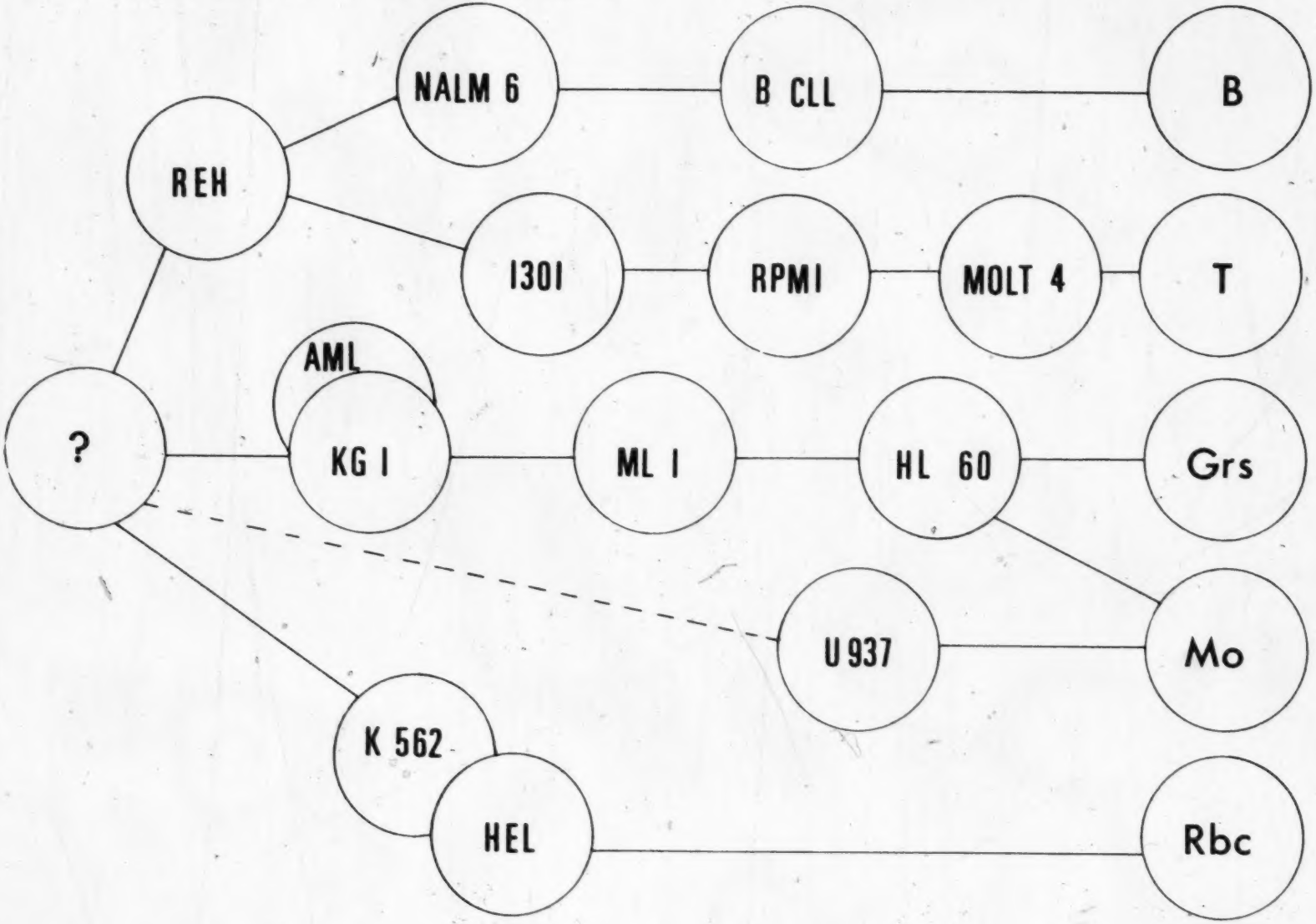


Table I. Main characteristics of the cell lines under study.

CELL LINES	ORIGIN	MAIN FEATURES	ENZYMES	SURFACE MARKERS	DIFFERENTIATION INDUCERS	EXPRESSION OF ONCOGENES
K 562	Blast crisis of CML	Undifferentiated blasts, Erythroleukemia	PK(L,M_1, M_2), alpha-napthyl esterase	no HLA glycophorin myeloid monocytic	Hemin, S. butyrate, PMA, INFs	C-erb C-abl
HEL	Secondary acute leukemia	Poorly differentiated blasts	PK(L,M_1,M_2), acid phosphatase butyrase	Class I Class II ($\pm$) glycophorin monocytic	TPA, DMSO, Hemin	?
KG-1	Acute promyelocytic leukemia	Myeloblasts	Chloroacetate esterase, acid phosphatase peroxidase	Class I Class II ($\pm$) myeloid monocytic	TPA	c-fos c-fes c-fms
HL-60	Acute promyelocytic leukemia	Myeloblasts, promyelocytes	Chloroacetate, esterase, myeloperoxidase	Class I no class II myeloid monocytic T-cell (T4)	DMSO, Retinoic acid, TPA, PHA-LCM, INFs, PGE_2	c-myc c-myb c-fos

1. THE HL-60 CELL LINE

The HL-60 cell line was established in 1977 by Collins from the peripheral blood of a woman with acute promyelocytic leukemia.

Constitutive HL-60 cells are positive to naphthol AS-D chloroacetate esterase, myeloperoxidase and Sudan Black B, stains being specific for granulocytic cells (17).

Phenotypically, most of the HL-60 cells appear to be arrested at the promyelocyte stage of differentiation (18,73). The process of differentiation and development of the HL-60 cells are influenced by a number of factors. This permits one to follow the changes related to proliferation, differentiation and cellular behavior (93). Different compounds can induce some differentiation. The most commonly used substances are DMSO, retinoic acid, TPA, interferons and many conditioned media.

A 1.3% solution of the polar solvent dimethylsulphoxide (DMSO) induces some promyelocytes to become bands and polymorphonuclear leukocytes (PMN), a passage which is accompanied by functional changes such as increased production of O_2^- (95), ability to

ingest, degranulate and kill microorganisms. However, it has been shown that these cells lack or bear defective secondary granules and that immature cells from the same cell line do not exhibit the above functions. Actually, the site and mechanism of action of DMSO and other compounds is unknown. DMSO is known to have several biologic activities, including the ability to solubilize membranes. It is possible that DMSO induces differentiation by altering cellular or subcellular ionic fluxes. Amiloride, a diuretic and passive inhibitor of Na^+ flux across the membrane, potentiates DMSO-induced differentiation of HL-60 cells (14). Amiloride may modulate cation fluxes of HL-60 that are important in the line's differentiation. However, definitive evidence on the mechanism of action of these agents is unavailable. DMSO and 1,25-dihydroxyvitamin D_3 induce cells to differentiate and express myeloid-specific nuclear antigens (92). The appearence of these antigens suggests cell maturation. HL-60 cells can also differentiate along the granulocytic pathway under the influence of retinoic acid (12).

In contrast, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment induces monocytoid differentiation.

Moreover, TPA increases the activity of the fluoride isoenzymes of non-specific esterase that are contained in the HL-60 cells (137). These isoenzymes are detectable in acute myeloid leukemias (CML) and have not been found in lymphoid or stem cell leukemias. In addition, TPA induces the expression of myeloid-monocyte antigens and increases cell adherence as well as the capacity to be induced to become adherent.

Interferons (IFNs) are regarded as part of the nonspecific defence system against viruses, some microorganisms, tumor and other abnormal cells. Since it is now known that IFNs affect immune responses, they can also be regarded as a class of lymphokines (interleukins). Alternatively, IFNs can be defined as multifunctional substances whose main roles are the control of virus and cell multiplication (55,114,115). Interferons are informational protein or glycoprotein molecules which in nanomolar concentrations interact with receptors on the outer surface of the plasma membrane of cells and trigger a cellular response. Thus, purified, recombinant DNA-derived gamma interferon is able to induce in the HL-60 cells morphologic, histochemical and functional changes, i.e. expression of antigens characteristic of monocytes and

granulocytes, monocytoid differentiation, induction of alpha-napthyl acetate esterase, increase in cell size and decrease in azurophilic granules (4). In the murine system, it has been shown that gamma interferon suppresses PGE_2 production but O_2^- release remains normal and therefore the macrophage suppressive activity is not affected (8). An analogous scheme may be true for HL-60 cells. The effect of gamma IFN on MHC antigens is not well understood. Constitutive HL-60 is class I positive and class II negative. All attempts to induce class II expression by gamma IFN are questionable (107).

Long term incubation of alpha and beta IFNs with myeloid cells alters their behavior towards different CSFs. This has been shown in mice (88) and similar phenomena have been observed in this work using HL-60 cells and recombinant gamma interferon.

A number of conditioned media (CM) have been used to induce differentiation. The effect of medium from phytohemagglutinin (PHA) stimulated lymphocytes (PHA-LCM) have been most fully described (15,40). These studies have indicated a decrease in RNA content and the differentiation to macrophage-like cells is accompanied by increased phagocytic capacity and

expression of alphanapthyl esterase. These macrophage-like cells are true adherent cells not found in the constitutive state. Similar events can be obtained by phorbol myristate acetate (PMA). PHA-LCM usually enhances hemopoiesis as is also the case for HL-60 differentiation. However, when PHA-LCM contains gamma interferon reverse effects are observed, i.e. it inhibits differentiation (139). PHA-LCM can also induce the expression of some new antigens and new surface glycoproteins.

Prostaglandins (PGs), and mainly of the E series, have been widely used in studies of HL-60's differentiation. PGs are a family of biologically active compounds generated from unsaturated fatty acids. They are produced locally in minute amounts and are metabolized almost instantly (38,99,136,140).

Some tumor cells can stimulate the production of large amounts of PGE by the monocyte/macrophage and since PGs also inhibit the production of lymphokines, which augment immune responsiveness and cytolysis, their production may be one mechanism responsible for the escape of tumor cells from immune surveillance (65,100). Prostaglandin E_2 is able to induce the proliferation of hemopoietic stem cells in vitro (32),

enhance erythropoiesis (27,34) and also drive HL-60's differentiation to mature granulocytes (14). In contrast, PGE almost totally inhibits colony formation for HL-60 in a mixed colony assay. This inhibition can be shifted by the addition of appropriate amounts of colony stimulating factor (65).

HL-60 expresses a number of oncogenes (cellular-genes, often analogous to viral transforming genes, that are directly responsible for neoplastic transformation of the host cell) such as the c-fos, c-myb and c-myc. The c-fos is detectable when HL-60 is induced to differentiate into macrophages by using TPA (91). This late stage of differentiation seems essential for the oncogene's expression (90). The view that c-fos is induced only when HL-60 cells differentiate to macrophages and not to granulocytes, is supported by the fact that when these cells are induced with DMSO no c-fos transcripts are observed (87).

The c-myb oncogene has been found to be expressed at the stage of precursor cells. Moreover, induction with DMSO or retinoic acid leads to decreased transcription of c-myb genes (128,129).

In general, the c-myc is an inducible gene, regulated by specific growth signals in a cell-cycle-dependent manner (59). Like the c-myb gene, c-myc is expressed at very early stages of development. Induction of differentiation by TPA (monocytic pathway) or DMSO (myeloid pathway) is accompanied by a decrease in the rate of transcription of the c-myc gene (43). Studies have shown that changes in c-myc expression in HL-60 cells subsequent to DMSO treatment can be attributed primarily to the cell differentiation process and not to a cell-cycle phenomenon (33). Alpha interferon has been found not to reduce the level of c-myc mRNA in HL-60 (16,29), whereas in the Daudi system (Burkitt lymphoma) it produces a five-to sevenfold reduction in c-myc.

2. THE KG-1 CELL LINE

The KG-1 cell line was established in 1978 from a 59 year old man with erythroleukemia that developed into acute myelogenous leukemia (64). The patient's bone marrow contained 30 percent myeloblasts and 10 percent erythroblasts with marked megaloblastic and dysplastic changes. This cell line retains some myeloid features in vitro and forms myeloid colonies in soft gel culture

in response to colony stimulating factors (64).

During exponential growth the majority of cells are arrested at the stage of myeloblasts and promyelocytes without clear terminal myeloid or monocytoid function. Analysis reveals 85% of cells positive for naphthol AS-D chloroacetate esterase, 98% positive for acid phosphatase, 52% positive for the Schiff reaction and 10% positive for peroxidase.

Retinoic acid and DMSO cannot induce the cell line and consequently no morphological changes can be observed after appropriate treatment.

Cell surface glycoproteins are also expressed. KG-1 has a small quantity of unbranched lactosaminoglycan. These amounts increase as cells mature from promyeloblasts to granulocytes (35). HLA class II antigens are detected on 20 percent of cultured cells (63). The presence of nuclear antigens is quite limited and observed only after induction with DMSO (92).

TPA can induce KG-1 cells to mature into non-dividing macrophage-like cells (61). Actually, low concentrations of phorbol diesters can enhance the growth stimulatory activity of CSF on these cells.

Human CSFs increase RNA synthesis (3 hours) and

after a lag of about ten hours, stimulate DNA and protein synthesis (70). Mouse CSFs, hormones and other factors do not trigger KG-1 cells.

Finally, some subclones of KG-1 have been reported. One of these, the KG-1a, retains the same constitutive markers but does not respond to CSFs and does not express the human Ia antigen (62). These subclones are useful for investigating cellular responses to CSFs.

3. THE K 562 CELL LINE

The erythroid cell line K 562 was originally established in 1970 by Lozzio and Lozzio from a pleural effusion of a woman with CML in blast crisis (68). At first it was believed to be a myeloid precursor arrested at an early stage of differentiation. This theory was based on 1) the tissue of origin, 2) failure to find detectable lymphoid surface markers, 3) the presence of a group specific granulocyte antigen and 4) the pattern of reactivity of K 562 heteroantisera with bone marrow and leukemic cells (60,69,25,131).

K 562 lacks ABH, Rhesus, Lewis, Duffy, P, P1, and Pk antigens. However, the "i" antigen is present, and its total amount can be increased after treatment/induction

with hemin (47,50).

On the other hand, a series of glycolipids having X-hapten structure in human erythrocytes are not immunologically detectable at the cell surface, whereas similar glycolipids having X-hapten structure in K 562 cells are highly expressed (57).

DMSO and sodium butyrate supress the expression of two myeloid antigens while hemin does not (49).

Attempts to induce granulocytic differentiation by DMSO (79) and retinoic acid (50) have been unsuccesful. In addition, no macrophage-type antigens have been detected on these cells.

In 1979, evidence was provided for the line's erythroid differentiation and it was demonstrated that it expressed glycophorin and spectrin (1). The line is able to synthesize globin chains when treated by hemin (86). The hemoglobin produced is of embryonic type, with the presence of small amounts of fetal hemoglobin. K 562 does not synthesize adult hemoglobins whether treated or untreated (44).

The erythroid markers are increased upon treatment with hemin or sodium butyrate whereas TPA significantly suppresses the expression of glycophorin and reduces hemoglobin synthesis (124).

Constitutively, K 562 is class I and class II negative, but gamma interferon and sodium butyrate are able to induce expression of class I antigens (117). Gamma interferon can also induce some hemoglobin synthesis while alpha and beta interferons can slightly augment the mRNA coding for beta-2-microglobulin (106).

Recent studies indicate that K 562 can express megakaryocyte markers after treatment with PMA. Moreover, the number of cells exhibiting peroxidase activity increases after sodium butyrate induction (119).

4. THE HEL CELL LINE

The human erythroleukemia cell line HEL was established from the peripheral blood of a patient with Hodgkins disease who later developed erythroleukemia. Cells are positive for periodic acid schiff (PAS), acid phosphatase and butyrase, stains detecting erythroleukemia, and negative for granulocyte-specific stains.

Recent work has revealed that DMSO and TPA can induce megakaryocytic differentiation. This is documented by the presence of 1) organelles morphologically resembling platelet alpha-granules, 2)

peroxidase activity and 3) platelet membrane proteins.

It has been found that TPA also induces morphological, functional and biochemical changes in HEL cells that are characteristic for macrophage-like cells. These induced cells exhibit enhanced adherence and phagocytic activity leading investigators to suggest an "unmasking by TPA of an inherent monocyte-like phenotype in the HEL cells" (102).

This cell line displays spontaneous and induced hemoglobin production, especially when stimulated by hemin. Production of hemoglobin Barts (Hb gamma 4) is observed and trace amounts of other embryonic hemoglobins as well (75).

The erythroid failure of the HEL cell line can be seen from studies on the pattern of pyruvate kinase (PK) isozymes. From the four forms (L,R,M_1,M_2),the R is characteristic for red cells and is found only in minute amounts in HEL cells. (The same applies for the K 562 as well) (76).

Other characteristics in common with the K 562 erythroleukemia are a) neosynthesis of ganglio-series glycolipids, b) suppression of branching in the core carbohydrate chain of lacto-series and c) suppression in synthesis of globo-series glycolipids (57).

The major drawback in studying cell lines is expressed by the lineage infidelity concept, i.e. the expression of markers of different lineages on the same cell (78,112). This observation leads investigators to classify cell lines to inappropriate categories or not to classify them at all. Lymphoid cell lines, on the one hand, do not exhibit infidelity and their phenotype show only minimal deviations or asynchronies (41). On the other hand, observations on myeloid and erythroid lines often support this concept either in the constitutive state or after treatment with an inducer of differentiation. The level at which these extra phenotypes occur is still controversial, since the expected finding that commitment to one lineage excludes the expression of markers of other lineages is invalid. One of the few accepted generalizations is that patients whose cells show infidelity are less responsive to therapy.

C) ASSAYS

Cell proliferation, differentiation and surface marker expression were approached using the following assays:

1) Cell proliferation / ^{3}H Thymidine incorporation:

A convenient method for quantitating cellular proliferation is to assay DNA synthesis (72,51,9,10). Incorporation of exogenous radiolabeled ^{3}H or ^{14}C thymidine (a precursor of DNA) into material precipitable by trichloracetic acid over a specified period of time is measured by scintillation counting.

HISTORY: The distribution of thymidine to chromatids of daughter cells was originally described in plant cells by Taylor et al (120) in 1957. In the early 60's, observations on cultured peripheral blood cells revealed similar events. The results indicated that thymidine which is incorporated to DNA of the chromosome is part of a physical entity that remains intact during successive replications and nuclear divisions. The basic chromosome is made up of two functionally complementary DNA-containing units which undergo replication to form four units. The units segregate in such a way that each daughter cell

receives an old and a new unit.

According to the Howard and Pelc's model (51) the cell cycle consists of four phases designated: G1, S, G2 and M. The synthesis of DNA occurs during the restricted period of mid-interphase, the S period. ^{3}H TdR incorporated into DNA is distributed to mitotic chromosomes of daughter cells in a "semi-conservative segregation".

Although long labeling (18 to 24 hours) minimizes the effects of asynchronous DNA synthesis, we used a four hour labeling time which is also sufficient to determine significant incorporation.

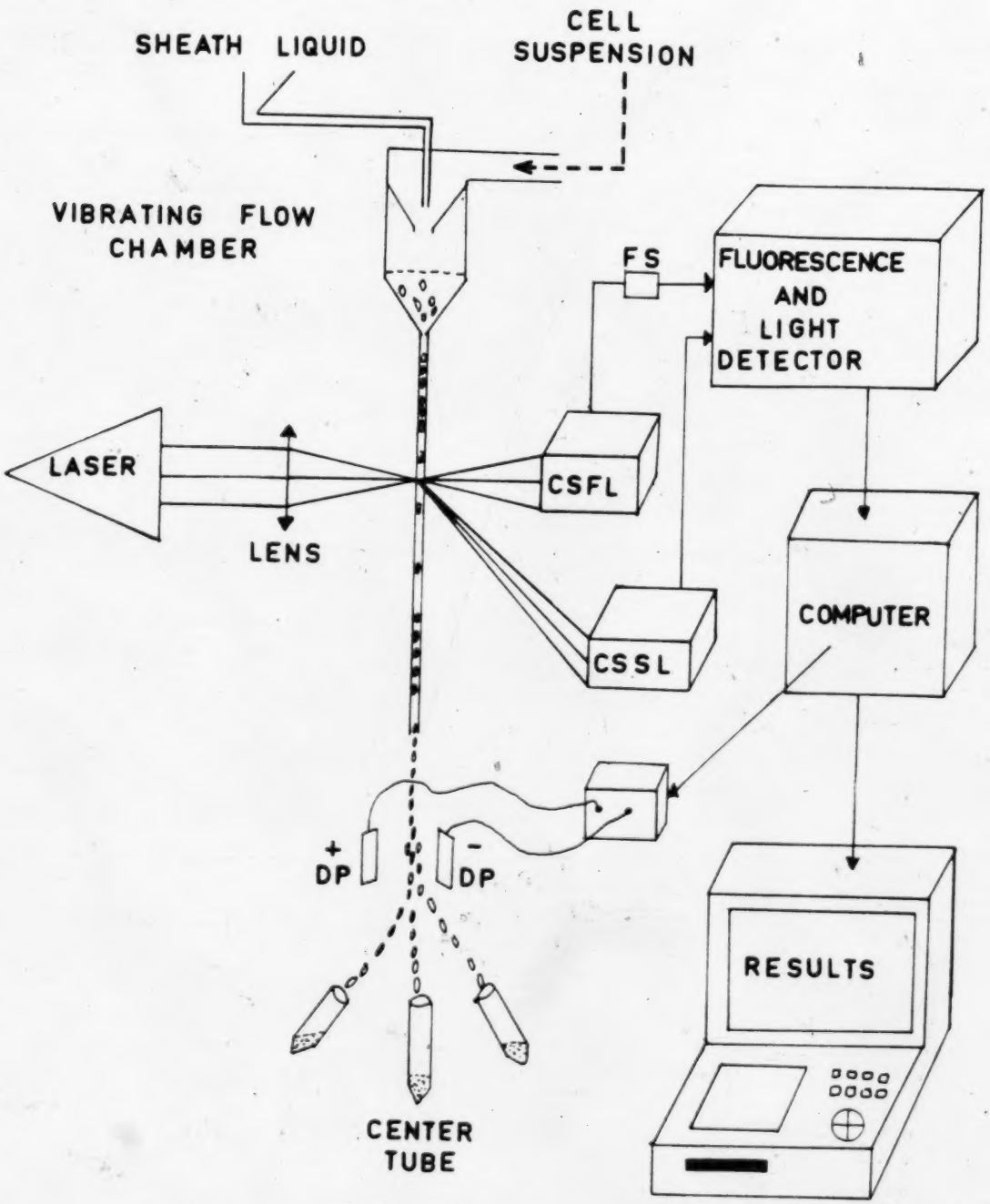
2) Surface marker expression / FACS analysis:

Determination of surface marker expression was accomplished with the use of a Coulter EPICS V flow cytometer using a 5 watt argon laser.

PRINCIPLE: The FACS operating principles will best be understood by reference to Fig.4, a simplified diagram of the instrument components.

The FACS system rapidly analyses and separates cells on the basis of fluorescence and light-scattering properties. This is accomplished by introducing cells in suspension to the center of a liquid stream and

Fig. 4
The FACS operating principles.



FS : FILTER SYSTEM

DP : DEFLECTION PLATE

CSFL : COLLECTING SYSTEM FOR FLUORESCENT LIGHT

CSSL : COLLECTING SYSTEM FOR SCATTERED LIGHT

causing them to pass, one at a time, through the focused beam of a high-power laser. In passing through the laser beam, a cell announces its presence by a characteristic scatter signal (which occurs for all cells) and by one or more fluorescence signals (which may also be generated). Each cell is individually characterized by the intensity, color, or polarization of fluorescence emitted while it is in the laser beam. These optical signals are converted by suitable photodetectors into electrical signals which are then processed by the instrument (48).

Cells that have been tagged by means of suitable fluorescent probes, or that exhibit native fluorescence can be detected by means of their emission when excited by the laser. When typical biological fluorochromes are used, excitation by the laser occurs at a selected wavelength, and fluorescence occurs at higher spectral wavelengths. This shift allows the use of selective optical filters to eliminate unwanted signals, and permits a cell to be identified solely on the basis of its affinity for the fluorescent material used. Excitation wavelengths range from 351 nanometers (ultraviolet) to 568 nanometers (yellow), enabling operation with a wide variety of fluorochromes.

3) Differentiation / Colony formation:

The colony assay, one of the major techniques used in this study, is very useful in defining groups of progenitor cells that behave differently in terms of self-renewal capacity, differentiation, proliferation and response to a number of factors. The depiction of hemopoiesis in the form of lineage diagrams, as described by McCulloch (78), is also based on these assays.

In this work, colony assays have been used for assessing differentiation only.

The first approach was developed by Till and Mc Culloch in 1961. They showed the existence of the pluripotent stem cell in the mouse system. This was also a confirmation of Pappenheim's theory established as early as 1900.

With time, a number of cell culture methods was described that permitted the growth of colonies in several media like in plasma clot, agar or methylcellulose. The viscous nature of the above systems was designed in order to prevent cell dispersion and allow the diffusion of beneficial or inhibitory substances.

Techniques for assaying committed progenitors and

pluripotent stem cells were then described. A system for generating granulocyte-macrophage colonies (CFU-GM) was described in 1965 (11,104). Six years later the erythroid colony forming units (CFU-E) system was developed (113). The erythroid burst forming units (BFU-E) that preceed CFU-E were described later by Axelrad et al. (2), and the method for CFU-Mk (megakaryocytes) followed (84).

Colony assays for multipotential progenitors, CFU-GEMM, which give rise to cells in the granulocyte, erythrocyte, macrophage and megakaryocytic lineages were developed by Fauser and Messner in 1978 (30,31). Recent observations suggest that CFU-GEMM represent relatively mature pluripotent progenitors (101).

CHAPTER II

MATERIALS AND METHODS

A. CELL LINES

1. CELLS: All the cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Confirmation of the exact identity of the cell lines was obtained by indirect immunofluorescent tests using appropriate panels of lineage-specific monoclonal antibodies. When in doubt, cytochemical assays were performed.

2. CULTURE CONDITIONS: All cell lines were grown in suspension culture in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Gibco Laboratories) containing different percentages of FCS - depending on the cell line - and supplemented with 0.05 mg/ml of gentamicin (Roussel Canada Inc., Montreal, Quebec). The cells were maintained at 37°C, in a humidified atmosphere, containing 5% CO_2. The cultures were diluted to low density (0.10 to 0.15 million cells/ml) three times weekly. Every four to six weeks, and depending on the cell growth, aliquots of the cells were frozen in liquid nitrogen for retrospective

analysis at a later time. Cell numbers and cell volume distributions were determined using an automatic cell counter, equipped with a channelyzer (Coulter Counter, model ZBI, Coulter Electronics Inc., Hialeah, Florida). Cell viabilities were assessed by trypan blue exclusion.

3. CLONING PROCEDURE: In an attempt to produce a gamma interferon resistant clone, the HL-60 Gallo culture was treated with 100 U/ml of recombinant gamma interferon. A few days later the culture was diluted to a concentration of ten cells per ml by serial dilutions. Aliquots (0.1 ml) of this suspension were distributed in individual microwells. Cells that proliferated and gave rise to cellular colonies after 8 to 14 days were picked and subsequently grown in large amounts of media. The sensitivity of the new sub-line to gamma interferon was tested by a colony assay as described elsewhere.

4. FREEZE-THAW PROCEDURE: Exponentially growing cells were concentrated by centrifugation to a final volume of 0.5 mls in RPMI 1640 containing 20% FCS. An equal volume of 20% dimethylsulphoxide (DMSO) in RPMI 1640 was added dropwise. The cells were cooled to -70^{o}C at approximately 1^{o}C per minute, and then stored in

liquid nitrogen.

The frozen vials were thawed rapidly by immersion and gentle agitation in a 37° C waterbath. The contents of the vials were transferred to a 12-ml conical centrifuge tube and diluted dropwise with 10 or 20% fetal calf serum in RPMI. The cells were centrifuged and then resuspended in 5 to 10 mls of RPMI 1640 supplemented with the appropriate percentage of FCS.

5. MYCOPLASMA TESTING: All the cell lines were regularly tested for mycoplasma contamination. The presence of mycoplasma was detected by two different techniques as described elsewhere (58,81). This work was done by Dr Chen, Dpt. of Medical Microbiology, U of A and Dr Koch, W.W. Cross Cancer Institute, Edmonton. All the experiments were performed with mycoplasma-free cell lines.

B. LONG TERM BONE MARROW CULTURES

1. BONE MARROW: The bone marrow (provided by the Dpt. of Surgery, U of A Hospitals, Edmonton, Alberta) was aspirated in preservative-free heparin from the iliac crest of healthy volunteers or the sternum of patients undergoing cardiac surgery. In both cases the marrow speciments were considered as normal ones.

2. INITIATION OF CULTURES: Long term cultures were of two types: a)Type 1 cultures were inoculated with cells from the unprocessed bone marrow aspirate (19,20) and incubated from the beginning at 33°C in a atmosphere of 5% CO_2 in air. After an initial three day period, all non-adherent cells were removed and layered over 1.077 g/cu cm Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, Md.). Red cells and mature granulocytes were discarded and light density cells washed and returned to their original dishes (Costar-6 well dishes, Johns Scientific, Ont.) in fresh medium. b)In type 2 cultures, marrow cells were diluted 1 : 2 in growth medium and separated on a Ficoll-Hypaque density gradient. Light density cells were washed two to three times and cultured at a final concentration of 2.5 million cells/ml. Both types of culture yielded identical results.

3. CULTURE CONDITIONS: The growth medium (19,20) consisted of alpha-Minimum Essential Medium (alpha-MEM, Gibco Laboratories, Grand Island Biological Company, Grand Island, N.Y.) supplemented with extra L-glutamine (400 mg/l), inositol (40 mg/l), folic acid (10 mg/l), horse serum (12.5%, Gibco), fetal calf serum (FCS, 12.5%, Flow Laboratories, Mississauga, Ontario), hydrocortisone sodium succinate (10^{-6}M) and gentamicin (50 mg/l, Roussel Canada Inc., Montrel, Quebec). Both sera used were heat-inactivated at 56°C for 1 hour, filtered, aliquoted and stored frozen prior to use (22,36).

The cultures were incubated at 33°C, in order to facilitate the development of the adherent layer and not to encourage high fibroblast formation, in an atmosphere of 5% CO_2 in air (23). Half of the medium was removed once a week or when judged by eye to be necessary and fresh medium was added. Viability tests were performed using the Trypan Blue method at regular time intervals.

4. FEEDER LAYER: Long term marrow cultures consist of two cellular compartments. The adherent cell population initially consists predominately of round cells, which during the third and fourth week develop

into enlarged cells with a fibroblast-like appearance and into cells with a lobulated surface (lipid containing cells). The non-adherent cells proliferate in close proximity to the adherent layer (46), and their relationship remains obscure (21).

5. SEEDING OF THE FEEDER: Three to six weeks after the initiation of the culture, the growth medium, which contained the non-adherent fraction, was totally removed and HL-60 or KG-1a cells were seeded at a concentration of 200 and 100 thousand cells/ml respectively. The new cultures contained 5% of FCS. The incubation was carried out at 37°C in an atmosphere of 5% CO_2 in air.

6. IRRADIATION OF THE FEEDER: We irradiated the feeder layer with 3,000 rads (36.2 minutes) in a Gammacell 40 irradiation device (Atomic energy of Canada, LTD.).

7. MOUSE FEEDERS: For comparative purposes some mouse feeder layers, provided by Dr M. Longenecker, Dpt. of Immunology, University of Alberta, were used in excactly the same manner as the human ones. They were derived from the D 10 and D 14 cell lines having a hepatic tumor origin (110,111). In addition mouse bone marrow from C3H mice was also used like its human

counterpart.

8. MONOCLONAL ANTIBODIES: We attemted to obtain a more profound understanding of the adherent layer's involvement by using a panel of monoclonal antibodies (Table II). Briefly, the antibodies were diluted in alpha-MEM, 5% FCS (1 : 50) and used in excess in order to bind all concerned sites on the cell surface of irradiated and non irradiated feeders. A first incubation was carried out at 37°C, 5% CO_2 for one hour and then the cells from the cell line were added at the concentration described above. After various times of incubation the proliferation was assayed by ^3H thymidine uptake.

9. COLONY STIMULATING ACTIVITY IN BONE MARROW SUPERNATANTS: The bone marrow supernatants were tested for colony stimulating activity using the method of Mosmann (89), modified by Dr L. Guilbert, Dpt. of Immunology, U. of A. In this very sensitive assay, reduction of the tetrazolium dye MTT reflects cellular metabolic activity.

Briefly, the macrophage MMS-47 cell line, developed by Dr L. Guilbert, was grown for two days at a concentration of 1,000 cells/microwell and tested for

Table II.

Monoclonal antibodies against the bone marrow feeder layer.

MoAb	Specificity	Origin
83H1	Class I	P. Mannoni
L11.12	Class II	M. Fellous
Maja 7	Class II	M. Fellous
81H5	Endothelium	P. Mannoni
82H1	Non-X	P. Mannoni
86H1	Stem cell	P. Mannoni
T4	T cell	ATCC
T8	T cell	ATCC

ability to reduce the tetrazolium salt MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Sigma chemicals, St. Louis, Mo.] to a formozan product.

For this, the MMS-47 cells were harvested in cold PBS, washed twice and then resuspended in Iscove's modified Dulbecco's medium (IMDM, Gibco, laboratories) containing 15% FCS and the supernatant(s) under test (final concentration 40 ul/well). 1,000 cells in 100 ul were added to each well (96 flat bottom well dish, Nunclon, Delta by Nunc, Denmark) and incubated for 48 hours at 37°C in 5% CO_2. By the end of the incubation period, the dishes were spun and the medium (IMDM-15% FCS) was removed. The cells were resuspended in 100 ul of MTT development medium containing one ml of MTT in water (5mg/ml) and ten mls of a solution composed by 5% FCS in IMDM supplemented by four mls of a conditioned medium - in this case 5637 CM. The dishes were incubated for 4 to 6 hours at 37°C. Then 100 ul of acid isopropanol (0.04 N HCl in isopropanol) were added, mixed well with the use of an Eppendorf pipet and read in an Elisa platereader (Titerek Multiskan, Flow lab.) using 570 and 630 nanometers as test and reference wavelengths respectively.

C. THYMIDINE UPTAKE

ASSESSMENT: Cell cultures were carried out a) in triplicate wells of V bottom microculture plates (Linbro, Flow Laboratories, Hamden, Connecticut). Twenty thousand cells in the presence of different CSFs/supernatants/interferon at a final volume of 200 ul were incubated at 37°C in 5% CO_2 in air for 48 to 96 hours, b) directly on the bone marrow feeder layer using the original dish in which the bone marrow culture was initiated. 100 and 200,000 cells for KG-1a and HL-60 Gallo respectively were seeded as described previously. 48 to 80 hours after incubation, 200 ul of each well were transfered to a microwell, and pulsed with thymidine (New England Nuclear, Ontario, Canada, spec. activity 20 Ci/mmol; 1 uCi/well). Four hours later, cultures were harvested onto glass fiber filters using a SKATRON (Flow) automatic cell harvester. The dried filters were added to scintillation vials containing 2-3 ml of Toluene with 1.38 g of Omnifluor/l and counted in a RackBeta 1218 (LKB, Wallac, Finland) counter.

Thymidine uptake is expressed as mean counts per minute (cpm) +/- standard error (SE) from triplicate wells.

Care was taken to avoid bacterial contamination since it can distort the results by causing excess label to be incorporated into the bacterial DNA.

D. INDUCTION OF CELL LINES

1. GAMMA INTERFERON: The use and activities of the main inducer, gamma IFN, have been well described in the literature. In preliminary experiments the optimal concentration that stimulated proliferation but did not interfere with cell viability as measured by trypan dye exclusion, was determined. Recombinant gamma IFN was used at concentrations ranging from 100 U/ml to 1,000 U/ml, depending on the cell line and/or test performed.

2. OTHER INDUCERS: The ability of several other agents to induce (mainly) class II expression on the cell surface was determined. They were:

a) Alpha and beta interferons.

b) F_3, CSF, IL-3, elaborated from the Phorbol Myristate Acetate treated EL-4 mouse cell line (6,103), provided by Dr C. Bleackley, Dpt. of Biochemistry, University of Alberta.

c) Bone marrow supernatants, that were collected at different time intervals (from three weeks to five months).

d) Supernatants elaborated from the contact between the bone marrow feeder layer and the HL-60 Gallo as well as KG-1a cell lines. These supernatants were also tested

for colony stimulating activity as it has been described previously.

e) The conditioned media PHA-LCM, HPCM, MoT, Mia PaCa and 5637, the preparation of which is described in the next section.

Determination of induction was assayed by FACS analysis (see below).

E. MEMBRANE EXPRESSION

FLOW CYTOMETRY ANALYSIS: The seeded and/or induced cells, after a certain period of incubation (2 to 5 days) were collected and examined for surface antigen expression by Flow Cytometry analysis using an EPICS V cell sorter (Coulter Electronics, Hialeah, Florida). The cells were washed twice with phosphate buffered saline (PBS) with 0.2% bovine serum albumin (Pentex Bovine Albumin 30% solution, Miles Scientific, Naperville, Il.) and were then resuspended in PBS, 2% FCS, 10% AB serum, 0.02% sodium azide solution. Fifty ul aliquots containing one million cells were mixed with 50 ul of diluted monoclonal antibody (MoAb) (Table III). After one hour at 4^{o} C, the cells were washed three times with PBS, 0.2% BSA and then 100 ul of a 1/50 dilution of fluorescein conjugated F(AB')2 fragment rabbit anti-mouse Ig G was added (Cappel laboratories, Cochranville, PA). The cells were incubated in the dark at 4^{o}C for one hour, washed as described previously and then resuspended in 0.5 mls of a 1% w/w formaldehyde solution (Formaldehyde solution, 37% w/w, Fisher Scientific Co.) in PBS. The samples were analysed by a Coulter EPICS V flow cytometer using

Table III.
Monoclonal antibodies used for membrane expression.

MoAb	Specificity	Origin
PBS	Neg. control	---
81H2	Class I	P. Mannoni
83H1	Class I	P. Mannoni
M18	Class I	M. Fellous
L11.12	Class II	M. Fellous
Maja 7	Class II	M. Fellous
7H3	Class II	M. Longenecker
Genox	Class II	ATCC
H81.98.7	Class II	M. Pieres
40.164.3	Class II	M. Pieres
72.202.2	Class II	M. Pieres
82.246.10	Class II	M. Pieres
40.315.7	Class II	M. Pieres
86H1	Stem cell	P. Mannoni
41H16	B cell	M. Longenecker
LFA-1	Surf. glycopr.	C. Mawas
82H5	Myelocytes	P. Mannoni
82H3	Monocytes	P. Mannoni
80H3	Mature myelocytes	P. Mannoni
39C15	IL-2 receptor	C. Mawas
39C65	IL-2 receptor	C. Mawas
33B31	IL-2 receptor	C. Mawas
33B73	IL-2 receptor	C. Mawas
T9	T cell	ATCC
T8	T cell	ATCC
T4	T cell	ATCC
BL4	T cell	P. Brochier

a 5 watt argon laser.

In these experiments, ten thousand cells of each sample were analysed for green fluorescence using the multiple data aquisition display system (MDADS). The gates were set, based on forward angle light scatter, to include both control and test samples, which may have changed in volume, and to exclude debris and agglutinated cells. Each test sample with its matched control was run consecutively to reduce error due to drift. Histograms showing the number of stained cells, as a function of the log fluorescence intensity were recorded. Controls were also prepared in parallel, i.e. non-stained cell suspensions for assessing the degree of background fluorescence.

F. COLONY FORMATION ASSAY

The ability of cells to form colonies in tissue culture is assayed in a single layer system, adapted from the soft agar-feeder layer technique (11,104).This method utilizes a solution of methylcellulose (54) instead of agar as the supporting medium. Moreover, the feeder layer is replaced by different conditioned media (see below). Two days before plating, the cell concentration in culture(s) was dropped to 500,000 cells/ml (109). The plated cells were thus at the begining of their exponential growth and therefore their ability to proliferate was increased.

The culture dishes contained the following ingredients: the cells under test immobilized in culture medium (alpha-MEM) with 0.8%, w/w, methylcellulose (Flow Methocel powder, visc. 4,000 cps), 5%, v/v, FCS, 10%, v/v, conditioned media and in some dishes gamma interferon (100 or more units per ml), IL-2 (100 U/ml), PGE2 (10^{-7}M) and hydrocortisone(10^{-6}M). (The number of the cells employed depends upon the cell line's ability to grow in liquid culture).

Usually, all the components were added to a Falcon plastic test tube in a total volume of three mls. This

was mixed well by vortexing and the suspension obtained was dispensed in one ml aliquots into Falcon plastic petri dishes using a syringe with a 18 G needle. The cultures were incubated at 37°C in 5% CO_2 in air and the colonies (more than fifty cells) were scored on day 6 (K562, HEL), 10 to 12 (HL-60 Gallo) and 14 (KG-1a), using an Olympus inverted microscope.

1. METHYLCELLULOSE: The methylcellulose was prepared (135) by dispersing 5.2 g of Methocel powder in 100 ml of hot (90°-100° C) autoclaved glass-distilled water. This resulted in a considerable volume increase. The suspension was allowed to cool, and 100 ml of cold double strength alpha-MEM was added. The resulting 2.5%, w/w solution was allowed to hydrate for 48 hours at 4°C. This stock solution was stored at 4°C for various periods of time up to one month, and was used in the cultures at a final methylcellulose concentration of 0.8%, w/w.

2. COLONY STIMULATING FACTORS: Colony stimulating factors (CSF) are required for the in vitro proliferation and differentiation of granulocyte-macrophage progenitors, and evidence is accumulating that the factors serve a physiologic role in the regulation of granulopoiesis (82). CSFs are produced by

a variety of tissues, but cells of the monocytic-macrophage lineage and activated T lymphocytes are prominent human cellular sources (71). Physically, the CSFs are quite heterogeneous, although much of the heterogeneity could be the result of aggregation or covalent modification of a common polypeptide chain.

2.a. HUMAN PLACENTAL CONDITIONED MEDIUM (HPCM): Human placentas are stored at 4°C and used within twelve hours of delivery. The outer membranes are removed and the placental tissues cut in 5 mm^3 pieces (82,97). After being rinced three times in Eisen's Balanced Salt Solution, six pieces are placed in each flat-sided tissue culture bottle containing 20 ml of RPMI 1640, with 5% FCS. The bottles are incubated for seven days at 37°C in a fully humidified atmosphere of 10% CO_2 in air. The conditioned medium is harvested and then centrifuged at 10,000 g for 20 minutes to remove debris and stored at -20°C. According to the literature, crude placental conditioned medium often contains inhibitory materials. In our experiments we did use crude HPCM at a final concentration of 10% without having any inhibitory effect.

2.b. PHYTOHEMAGGLUTININ-LEUCOCYTE CONDITIONED MEDIUM (PHA-LCM): Conditioned medium from peripheral

leucocytes exposed to 1% PHA was prepared according to the method of Aye (3,37). Briefly, 200 ml of peripheral blood (Canadian Red Cross, BTS, Edmonton) was allowed to sediment at unit gravity in a 2 : 1 mixture of dextran 60 (Abbot Laboratories, Chicago, Il.) for 90 minutes. The cells in the supernatant were removed and washed twice by centrifugation at 500 g for ten minutes. They were resuspended at one million per ml in RPMI 1640 medium, supplemented with 20 mM L-glutamine, 1% vitamins, 1% penicillin/streptomycin (10,000 U/ml), 15% FCS and 1% PHA (Sigma, St. Luis, Mo.), and cultured in 5% CO_2 at 37°C for seven days. The supernatant was collected following centrifugation and the conditioned medium was filtered through a 0.45 um membrane (Nalgene, Rochester,N.Y.) and stored at -70°C until use. A 10% final concentration was used for the platings.

2.c. Mo T: This conditioned medium comes from the supernatant of the Mo T cell line which was established in 1978 with spleen cells from a patient with a T cell variant of hairy-cell leukemia (39,71). The cell line grows in alpha medium with 20% FCS and 10^{-4}M alpha-thioglycerol and it continuously releases a potent CSF into the supernatant, which is finally spun, filtered

and stored at -20°C. The preparation used for the experiments was unpurified and the optimal concentration was found to be 10%.

2.d. MIA Pa Ca: MIA PaCa-2 is a human pancreatic carcinoma cell line (138) which when in culture elaborates the relevant conditioned medium which is used at a final concentration of 10%. The initial MIA PaCa-2 culture was established in 1976. Briefly, a piece of tumor of about 2 cm in diameter was divided into two segments, each of which was placed in a 100 mm Falcon tissue culture petri dish and minced with a pair of sharp scissors into 1 to 2 mm piecies. The tissue fragments in each dish were dispersed in 5 ml of Dulbecco's modified Eagle's medium containing 10% FCS and 2.5% horse serum (DME-HSFCS), and incubation was carried out at 37°C in 5% CO_2 in air. Serial transfers of primary and established cultures was carried out by trypsinization using 0.1% trypsin in PBS. The MIA PaCa conditioned medium is obtained by centrifugation and filtration of the supernatant. The crude preparation we used was stored at -20°C.

2.e. 5637: The bladder carcinoma cell line, 5637, produces CSF(s) for myeloid colony growth from both normal and malignant (CML) bone marrow (94). The 5637

conditioned medium has also been found to be active in stimulating the growth of mixed granuloerythropoietic colonies from murine bone marrow. In some instances it is quite potent so that it can replace PHA-LCM. Its production requires incubation of 5637 cells (500,000/75 cm^2 flask) with 15 mls of alpha-MEM supplemented with 10% FCS. After 8 to 15 days of culture the supernatant medium, containing some free-floating non-adherent cells, is removed, centrifuged to remove cells and debris and filtered through a 0.2 um Millipore filter. This conditioned medium retains its potency for a number of weeks when stored at $4^{\circ}C$. For longer storage, at $-20^{\circ}C$, the conditioned medium has to be concentrated. The final concentration used was 10% but it is active between 5 and 10%.

3. PROSTAGLANDIN E 2 (PGE_2): PGE_2 was purchased from Sigma Chemicals, St. Louis, Mo. and used at a 10^{-7}M concentration.

4. INTEREUKIN-2 (IL-2): Human, recombinant IL-2 (6) was provided by Chiron Corporation-Emmersville, CA. and used at a final concentration of 100, 500 and 1,000 U/ml. As control, purified JURKAT supernatants containing the IL-2 fraction were also used at the same concentration. This was a gift from Dr C. Bleackley,

Dpt. of biochemistry, University of Alberta.

5. INTERFERONS: Human, recombinant alpha, beta and gamma interferons were obtained from Dr E. Falcoff and J. Wiezerbin (Institute Piere et Marie Curie, Paris, France). One hundred to one thousand units per ml were used for different experiments.

6. MONOCLONAL ANTIBODIES (MoAb): The T4 and T8 MoAbs were tested on the colony forming capability of the HL-60 cell line, at a final concentration of 1:10 and 1:25.

7. RECLONING: Since gamma IFN greatly inhibits colony formation, we attempted to obtain a sub-clone of the HL-60 Gallo line resistant to gamma IFN. For this, cells were treated with gamma IFN before recloning and individual colonies were picked and placed in microwells with 100 ul of growth medium. The colonies that gave rise to a considerable cell population were tranfered to flasks and incubated according to standard culturing procedure. These cells were tested again in a CFU-assay, by adding gamma interferon, to determine whether their colony formation was resistant to the effects of this agent. The same cells were also tested for surface marker expression.

G. THE C-MYC ONCOGENE

Many tumor viruses carry one or more genetic loci that are directly responsible for neoplastic transformation of the host cell. These viral oncogenes probably do not change the genetic material of the host cell.

It has been reported that the c-myc is an inducible gene regulated directly by growth signals which promote proliferation and expression in a cell-cycle dependent manner (59).

We decided to treat the HL-60 Gallo cell line with 100 U/ml of gamma interferon, which was added on days 0, 3, 5, and 7, for two cycles and then at a dose of 200 U/ml for another two cycle period. Experimental and untreated cells were taken out on each of these days for a CFU-C assay and thymidine incorporation. Moreover, 3 million cells were used each time for extraction of mRNA and subsequent dotting in order to determine the expression/presence of the c-myc oncogene on these cells (see below). The probe for the c-myc was a gift from Dr Daniel Birnbaum (CSH Lab. Dr Wigler).

1. CELL LYSATES: The cells were pelleted by centrifugation (600 xg, 5 min.), resuspended in one ml of PBS and repelleted by centrifugation in a 1.5 ml

tube (15,000 xg, 15 sec. in an Eppendorf centrifuge). The supernatant was removed by suction and the pellet resuspended by vortexing (130). 45 ul of ice-cold 10 mM Tris (pH 8.0), 1 mM EDTA were added to resuspend the cells, which were subsequently lysed by addition of two 5 ul aliquots of 5% Nonidet P-40 (Shell chemicals) with 5 minutes of mixing on ice in between. Following pelleting of nuclei (15,000 xg, 2.5 min), 50 ul of the supernatant were transfered to a 1.5 ml tube containing 30 ul of 20 X NaCl/Cit [0.15 M NaCl/0.015 M trisodium citrate (SSC : Standard Saline Citrate)] plus 20 ul of 37%, w/w formaldehyde (for denaturation of the samples). The tubes were incubated at 60°C for 15 minutes and stored at -70°C.

2. DOTTING: Dotting (125) single sheet of nitrocellulose, hybridization with a cDNA probe corresponding to c-yc, autoradiography and scanning (130,125) were performed by Dr C. Bleackley, Dpt. of Biochemistry, University of Alberta. Briefly, Schleicher and Schuell minifold filters (S & S, BA 85) were soaked in water for 5 minutes and then in 15 X SSC for more than one hour.

5 to 20 ul of each sample were serially diluted with 15 X SSC in a 96-well microtiter platet to yield a

final volume of about 150 ul which were applied with suction (3 minutes for 150 ul) to a 4-mm diameter spot on the nitrocellulose sheet (precut 4" x 5" 1/4), supported by a wet with 15 X SSC backing sheet (S&S # 470). The nitrocellulose was then baked (80°C, 90 min.) in a vacuum oven to fix cytoplasmic macromolecules.

CHAPTER III

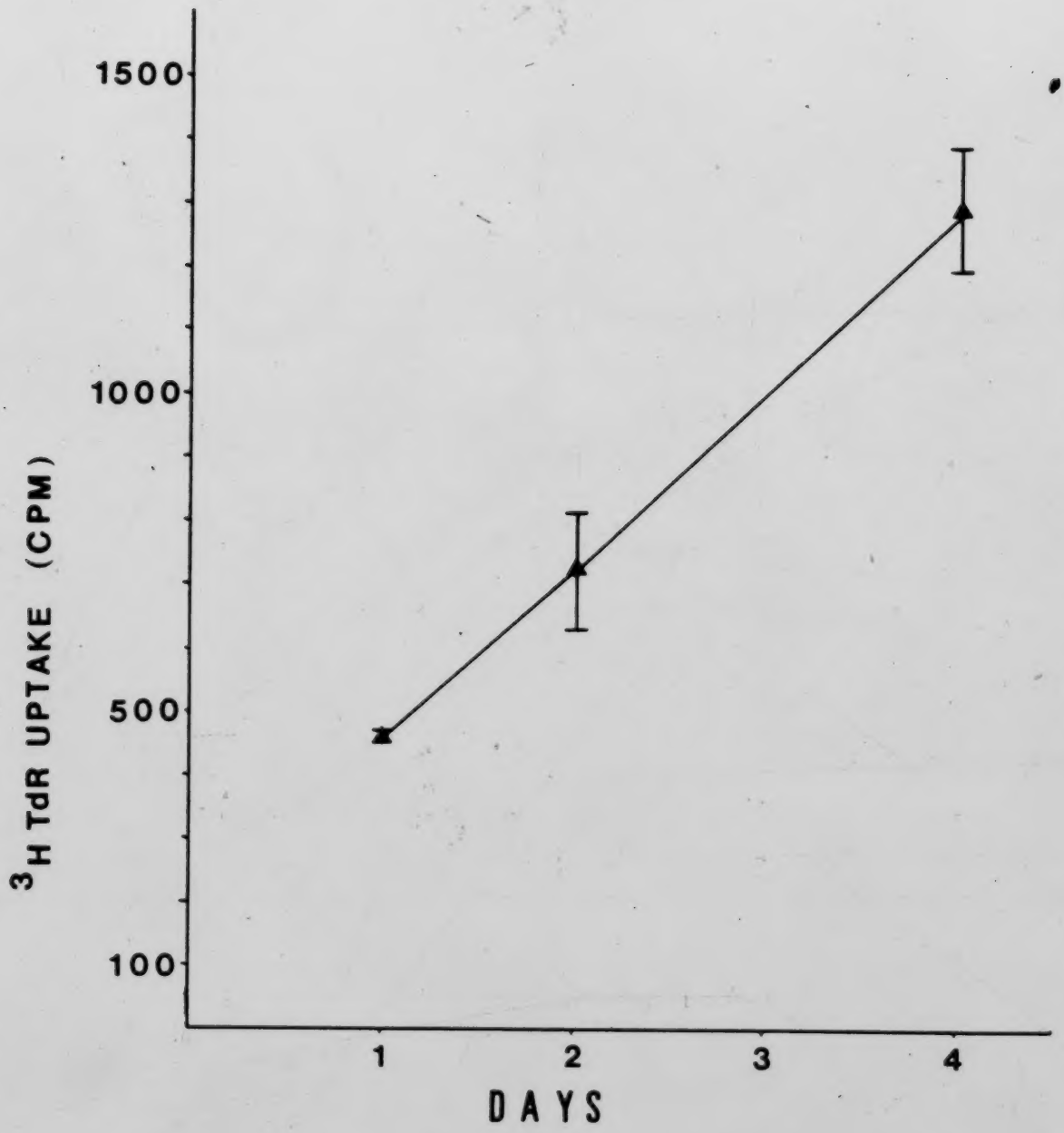
RESULTS

The proliferation, differentiation and surface antigen expression of HL-60, a promyelocytic leukemia cell line, has been studied. The majority of the cells of this line are promyelocytes but they can be induced along the myeloid pathway with DMSO or the monocytic pathway with TPA. Other (chemical and/or biological) compounds have been reported that can drive the line along the same pattern.

A. PROLIFERATION

HL-60 Gallo cells were grown in RPMI 1640 medium supplemented with 20% FCS and 0.05 mg/ml of gentamicin. Their doubling time was approximately 36 hours, as can be seen from Fig. 5. Attempts to increase the proliferative capability of the line with the use of a number of growth factors and/or biological compounds were unsuccessful. The human growth factors used were the Human Placental Conditioned Medium (HPCM), Mo T, PHA-LCM, 5637 and Mia PaCa. In a timing test only HPCM stimulated division producing a two-fold increase in proliferation after four days in culture as assessed by

Fig.5
Proliferation pattern of the HL-60 cell line.



^{3}H Thymidine incorporation. A summary of these events is given in Fig. 6. Gamma interferon, IL-2 and some mouse derived lymphokines from the PMA activated EL-4 cell line did not cause significant stimulation. In parallel experiments, the ability of various MoAbs namely 81H2 (class I), 83H1 (class I), L11.12 (class II), M18 (beta-2-microglobulin), T4 and T8 to cause proliferation in a five days test was assessed. None of the above antibodies was able to increase proliferation. On the contrary, observations show that a cell to cell interaction can very significantly stimulate proliferation. In fact, when HL-60 cells were added on the top of a human bone marrow feeder layer (hbmfl) and incubated for 48, 60 and 80 hours, their growth increased. Fig. 7 demonstrates these events. The effect of gamma interferon on the same system was negligible. To test the proliferating population, we irradiated the feeder with 3,000 rads for about 35 minutes. The results indicated that only the seeded cells were growing over the bmfl. KG-1a cells were similarly stimulated to proliferate when seeded on the same system.

The bone marrow feeder layer is composed of endothelial-like cells and of fatty cells. The first

Fig.6
Factors affecting HL-60's proliferation.

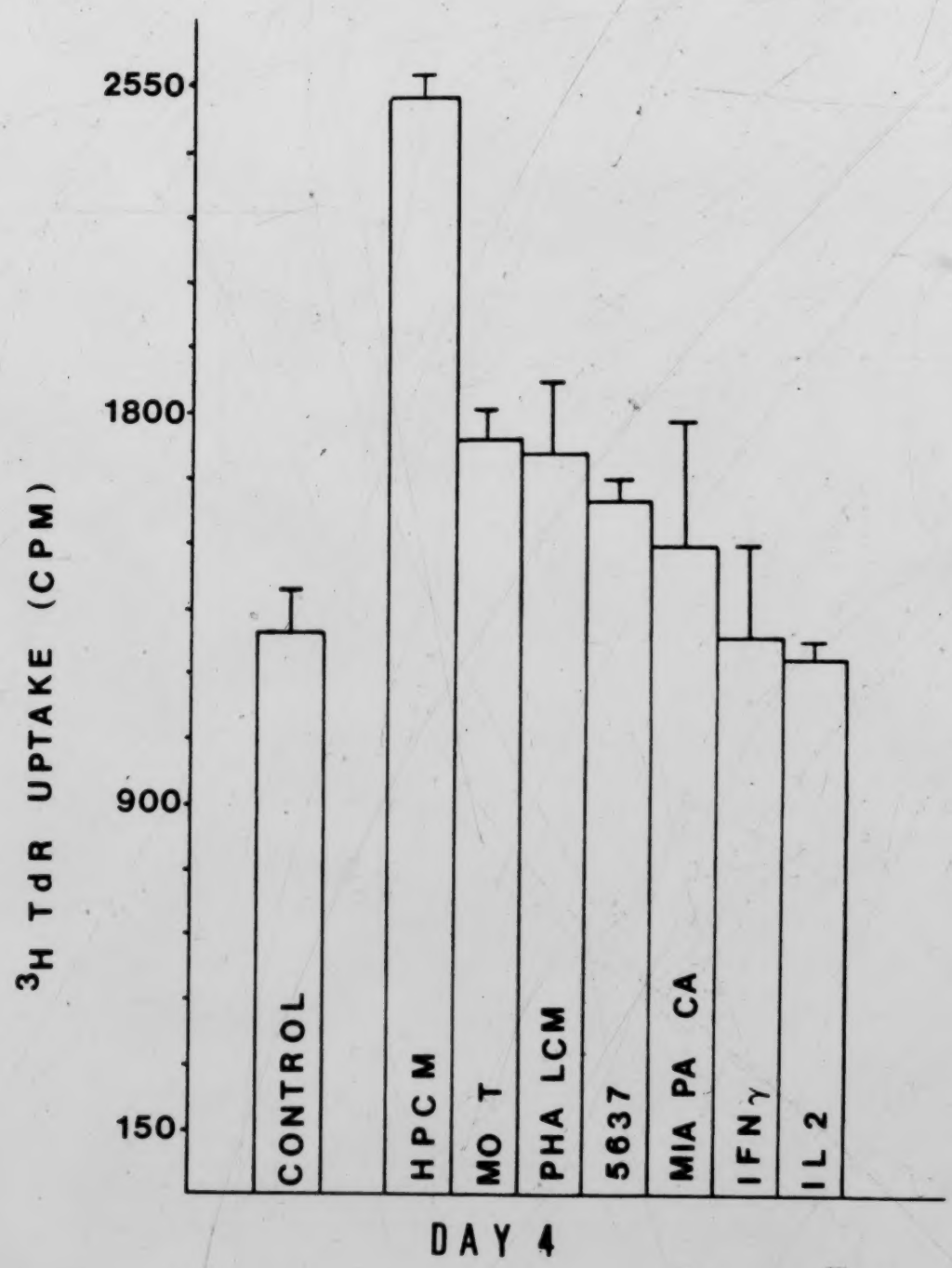
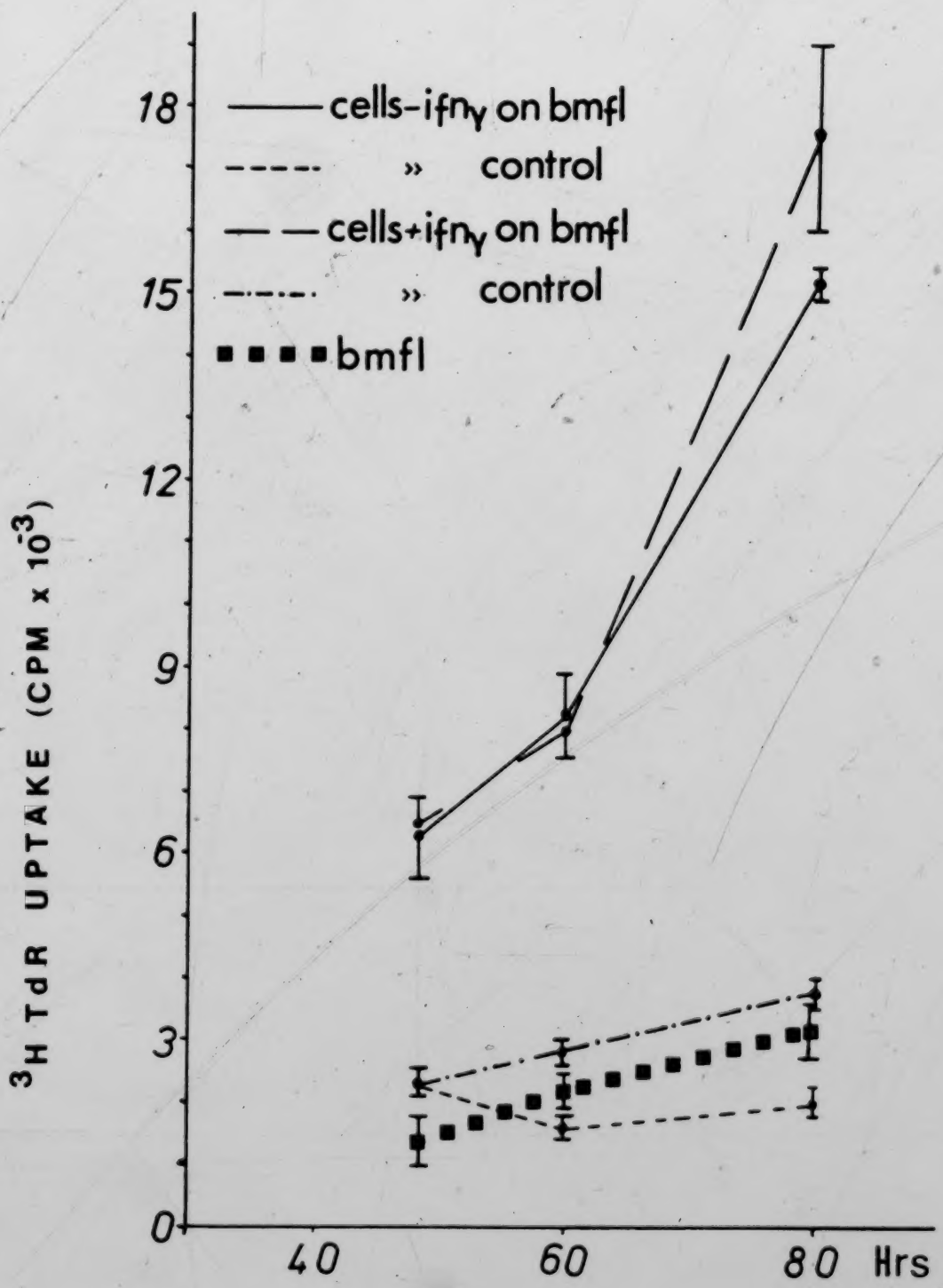


Fig.7
Effect of HBMFL on leukemic cell (HL-60) growth.



adherent cells appear after three weeks of culture and by the fifth week confluence is achieved. Beyond this point the layer becomes thicker and the fatty cells predominate. These three dinstict phases of the bmfl growth is shown in Plates 1 to 3.

We were able to maintain the life of the bmfls up to six months. Functional tests showed that after four months in culture they were not able to support the same, or a similar, proliferative response. When the direct cell to cell contact was interrupted by inserting a thin layer of methylcellulose between the proliferating cells and the feeder layer a considerable decrease of the thymidine uptake pattern was observed, showing a direct cell interaction between the two populations. In support to this view, supernatants of different human bone marrow cultures were unable to augment counts over background. This coincides with the finding that those supernatants showed only trace amounts of CSF-GM as assessed by the MTT reduction experiment.

We employed a panel of MoAbs (Table II) against the bmfl in an attempt to define the cellular structures involved in this interaction. Inhibition above 45% was obtained with the 81H5 (endothelial),

PLATE 1
Initial stage of bone marrow feeder layer formation (x40).

PLATE 2
Establishment of adherence (x40).

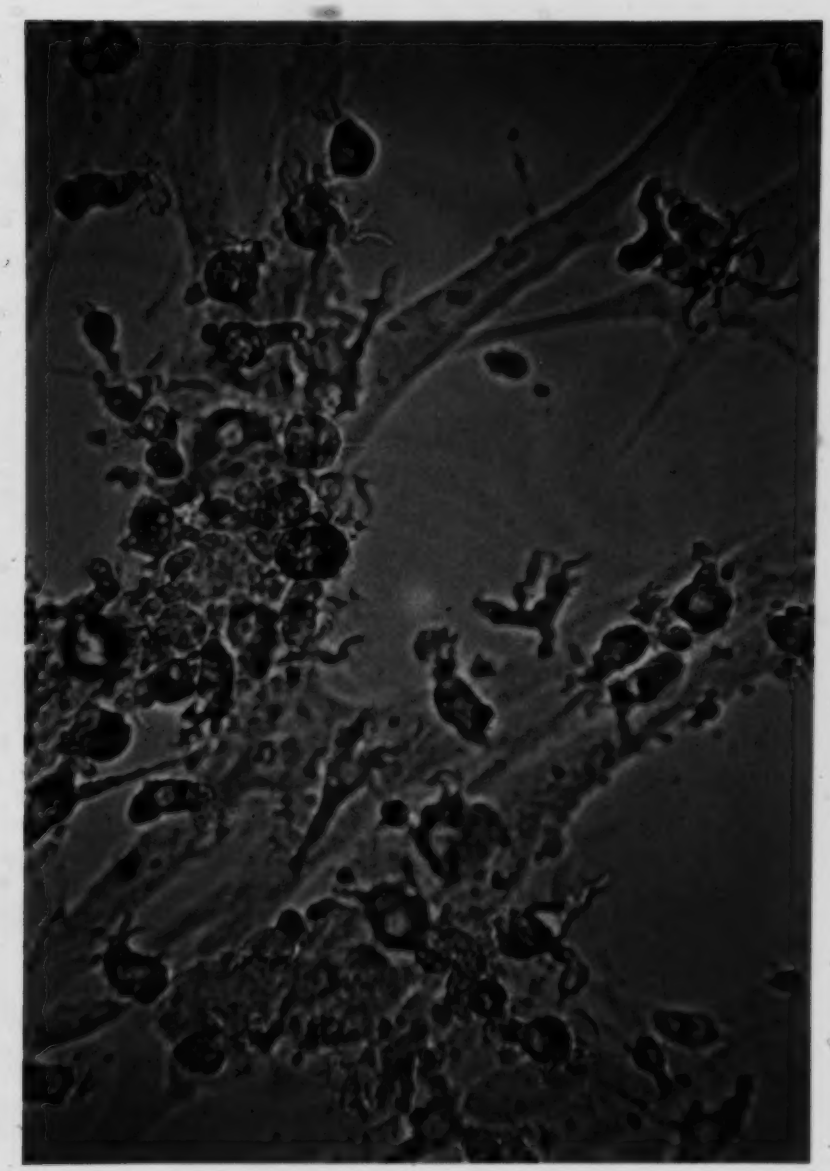
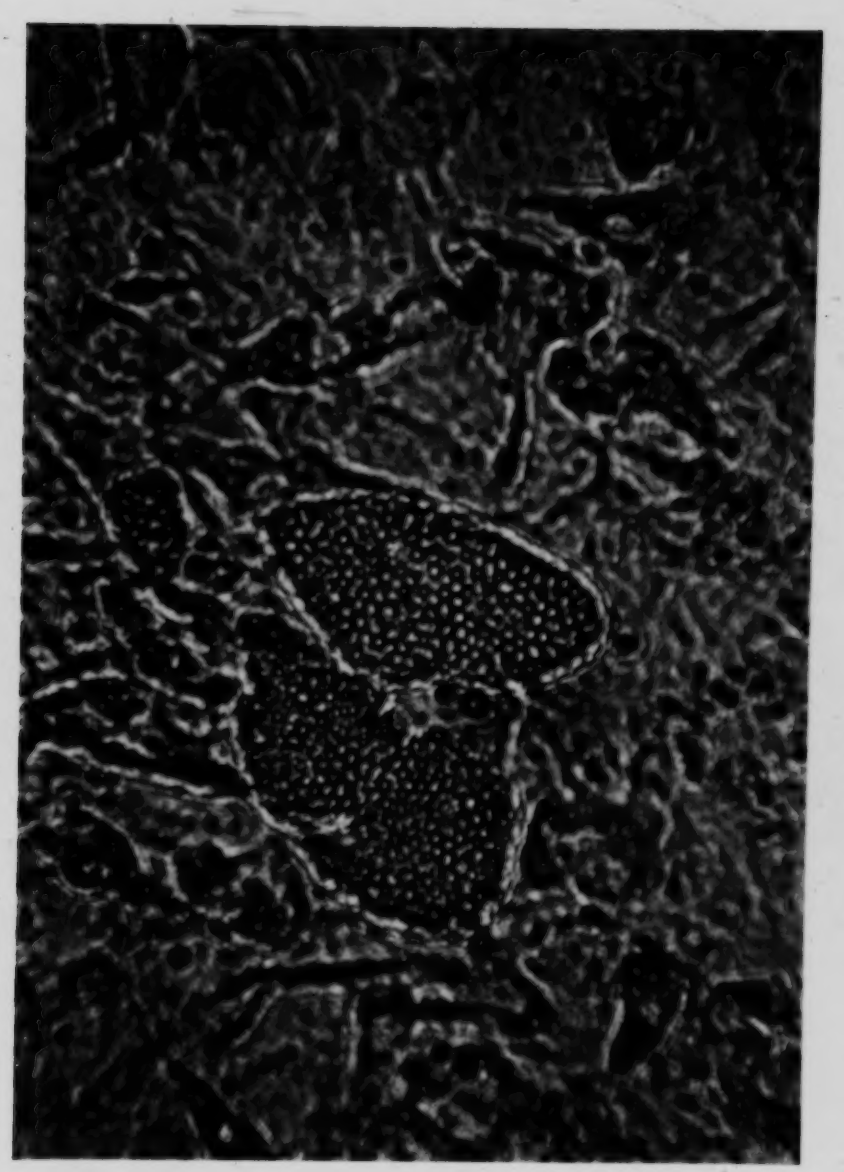


PLATE 3
Appearence of fatty cells (x40).



83H1 (class I), L11.12 (class II) and 82H1 (Non-X) MoAbs (Table IV).

Bmf1 similarly supported the growth APML (acute pro-myelocytic leukemia) cells. These cells resemble HL-60 cells in many aspects. This feeder layer system also supports the growth of normal bone marrow cells (light density cells depleted of monocytes) in a similar manner.

In another series of experiments, HL-60 cells were placed on the top of a mouse bone marrow feeder layer (mbmf1). This layer did not support growth, nor did the mouse endothelial cell lines D 10 and D 14. These observations suggest that a species-specific interaction is probably required to obtain proliferation.

Table IV.
Effect of MoAbs on bmfl related to HL-60's proliferation.

^{3}H TdR uptake (cpm)*

MoAb	0 Rads	% INH.	3,000 Rads	% INH.
Neg. control	16,482±2,026	—	13,750±353	--
81H5	10,168±856	30	7,290±798	47
83H1	11,017±168	24	7,068±93	48
Maja 7	10,428±224	28	12,613±1,399	17
L11.12	14,026±663	3	7,290±1,001	47
82H1	5,064±260	65	7,458±502	46
T8	5,907±109	60	11,822±274	23
T4	9,840±487	32	9,982±645	27
86H1	8,230±169	43	7,948±492	42

* : After three days of culture.

B. SURFACE MARKERS

Constitutive HL-60 is negative for histocompatibility antigens of class II but strongly positive for class I. It is also positive for myeloid markers while it is negative for markers for promonocytes and mature monocytes. The T4 antigen is expressed on these cells whereas other T cell markers are not expressed. These antigenic characteristics change when the cell line is induced by gamma interferon. For class I antigens the observed increased percentage of positive cells is quite peculiar. The observations recorded in Fig.8, show that untreated HL-60 cells, but tagged with a class I MoAb (83H1), give a single curve representing an almost totally positive cell population. However, when these same cells are treated with gamma IFN and labeled with the same MoAb, two peaks are observed on day five, suggesting an increase of the fluorescent intensity of some cells despite the fact that the absolute numerical values are about the same (94 and 92%).

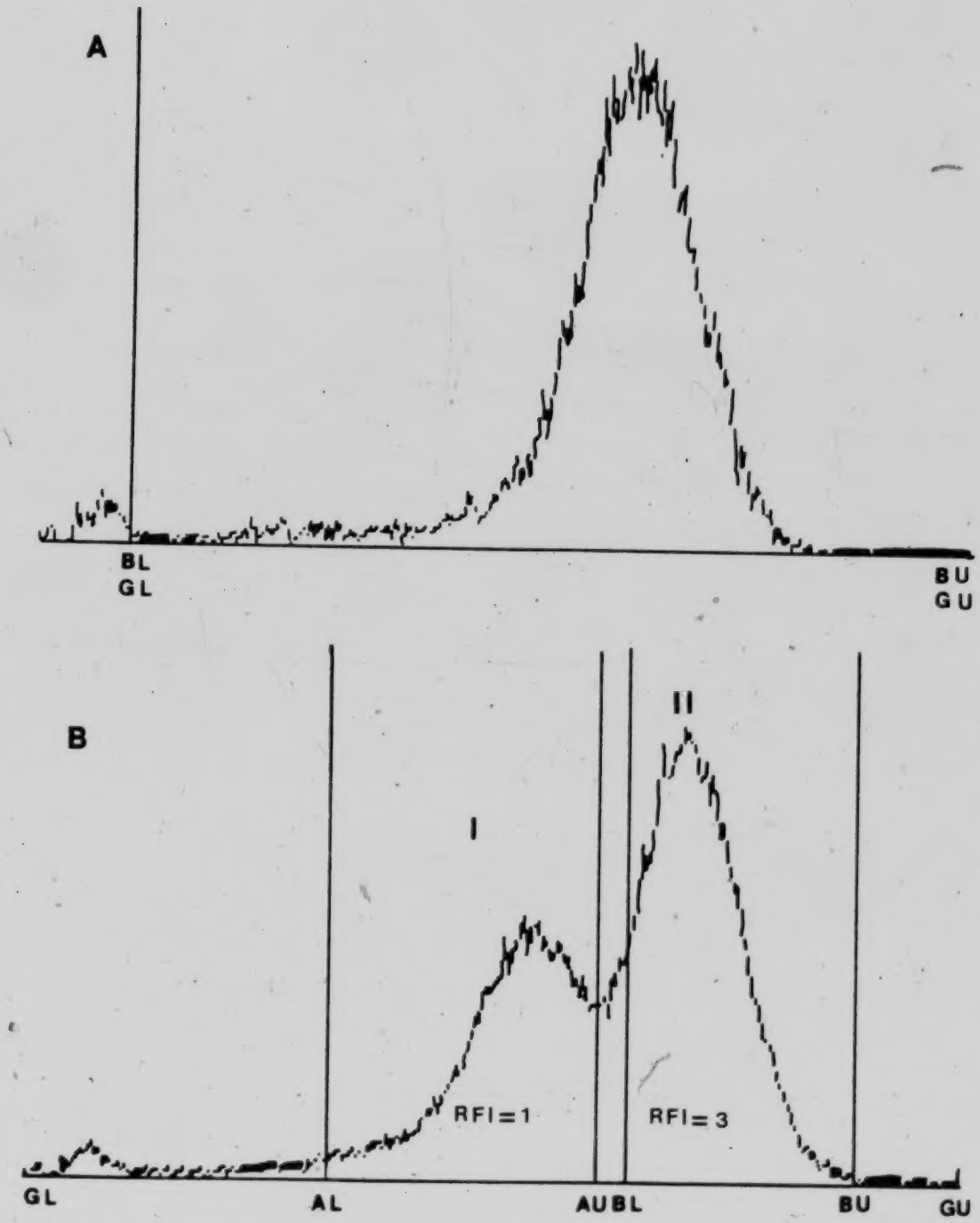
Applying the formula of Weeks (126),

$$RFI = 2^{\left(\frac{\text{I.M.F - Not I.M.F}}{25.6}\right)},$$

Fig.8
Class I induction after a five day treatment with gamma IFN.

A: Untreated HL-60 cells stained with the class I MoAb 83H1.

B: The same cell population after gamma IFN treatment and labeled as above.



where RFI is the relative fluorescence index, I.M.F is induced mean fluorescence and 25.6 a measure of the resolution of the machine, we can see that the right peak, compared to the left one, shows a population almost three times more positive for class I. Attempts to plate the two sorted populations of HL-60/gamma interferon treated cells revealed that the population expressing less class I antigen (peak I) gives more colonies than the more positive one, the ratio being 10 to 1. An explanation for this is difficult as the mode of action of gamma IFN is still unknown. On the other hand, gamma IFN can induce class II expression as detected with the L11.12 and Maja 7 MoAbs. This expression is questionable since a battery of other class II monoclonals gives an expression ranging from 10 to 84% (106).

Incubation of HL-60 cells with alpha and beta interferons has little if any effect. Table V summarizes the constitutive and induced expression of antigens on HL-60, including surface markers of other cell lines for comparison. The conditioned media used throughout this work did not induce class II expression but enhanced the binding of the 82H3 MoAb, specific for promonocytes and monocytes (Table VI). Since 5637, HPCM

Table V.
Surface marker expression after treatment with gamma IFN.

In fact, the actual % values for the MoAbs (class II) within the arrow area were ranging from 10 to 84 %. In this table, the higher percentages are reported.

	HL-60 Gallo				KG-1a	HEL	K 562
MoAb	Control	γ IFN	β IFN	α IFN	Cont.	Cont.	Cont.*
Neg. control	2	7	4	4	4	6	5
81H2	91	97	98	98	46	84	24
83H1	94	92	98	98	37	76	5
M18	93	98	99	99	34	61	7
L11.12	3	86	28	7	54	7	4
7H3	2	51	4	5	77	6	5
Genox	1	10	2	2	7	4	5
H81.98.7	2	84	5	3	68	5	4
40.164.3	2	73	5	4	39	5	3
72.202.2	1	60	3	3	69	5	3
82.246.10	1	27	4	4	51	6	4
40.315.7	2	78	4	3	4	13	3
Maja 7	2	63	4	3	23	5	ND
86H1	83	46	78	65	55	43	66
41H16	3	74	5	4	49	13	64
LFA-1	26	48	40	18	94	6	ND
82H5	95	98	98	99	55	ND	ND
82H3	8	50	40	33	26	37	66
80H3	2	26	3	2	6	ND	ND
39C15	2	3	2	2	3	4	2
39C65	2	68	6	2	14	5	2
33B31	2	3	3	3	18	4	3
33B73	1	3	2	2	15	4	2
T9	2	5	2	3	48	4	18
T8	5	5	ND	ND	ND	ND	ND
T4	72	88	ND	ND	ND	ND	ND
BL4	70	81	ND	ND	ND	ND	ND

ND : Not Done
* : Percentage of positive cells after a four day induction.

and Mia PaCa conditioned media are IFN free (Dr Wiezerbin, work in progress), there must be other regulatory molecules present in these media that can cause an increase in expression of the antigen recognized by 82H3.

In addition, the 5637 CM and the HPCM gave an increased class I expression resembling to the one obtained by gamma IFN. Actually, these media induced expression of HLA class I antigens as observed by a right shift of the second peak. However, this increase is less strong than the one obtained by gamma IFN.

Gamma IFN was also able to induce expression of the 41H16 marker, a B cell marker, on HL-60. This MoAb was developed against hairy cell leukemia and recognizes a 19,000 MW protein molecule on activated B cells and B cell malignancies.

The expression of the T4 antigen was not enhanced by gamma IFN or CM as seen by the formula of Weeks.

Supernatants of various sources as well as mouse factors could not induce any further expression.

Table VI.
Surface marker expression after induction with various conditioned media.

	Percentage of HL-60 Gallo positive cells *					
MoAb	Control	HPCM	PHA-LCM	Mo T	Mia PaCa	5637
Neg. control	3	4	4	1	8	6
83H1	92	99	99	99	98	99
M18	96	98	99	99	95	98
L11.12	3	5	3	3	3	4
7H3	1	2	4	6	3	4
82H3	8	80	84	79	54	76
39C15	2	6	4	5	8	4
39C65	3	3	2	2	2	1
33B31	3	2	2	2	5	3
BL4	72	63	65	65	68	70

* : Tests performed after a four day induction.

C. DIFFERENTIATION

The differentiation pattern of the HL-60 and other cell lines was studied by means of colony assays.

Despite the fact that three kinds of colonies have been reported in the literature, we only scored the compact ones and excluded those having a tight center with loosely defined outer boundaries and the loosely dispersed type. The exclusion of the above types of colonies does not actually affect the results, since they represent a 4% of the control and a 6% of the stimulated colonies. It is also worth noted that after gamma IFN treatment, only compact-type colonies are formed.

Plates 4 to 9 illustrate some different types of colonies obtained after treatment with gamma IFN and HPCM. It can be clearly seen that their morphology changes according to the type of factor added.

Gamma interferon had a suppressive effect on the colony formation of HL-60 cells (Plates 4, 5), which is dose dependent. The most effective concentration was 500 U/ml since it caused 90% inhibition. Gamma IFN acted similarly on the colonies formed by KG-1a which is another myeloid line having a lower plating efficiency (Plates 6, 7 and Fig. 9). This factor was

PLATE 4
HL-60 control colonies (x10).

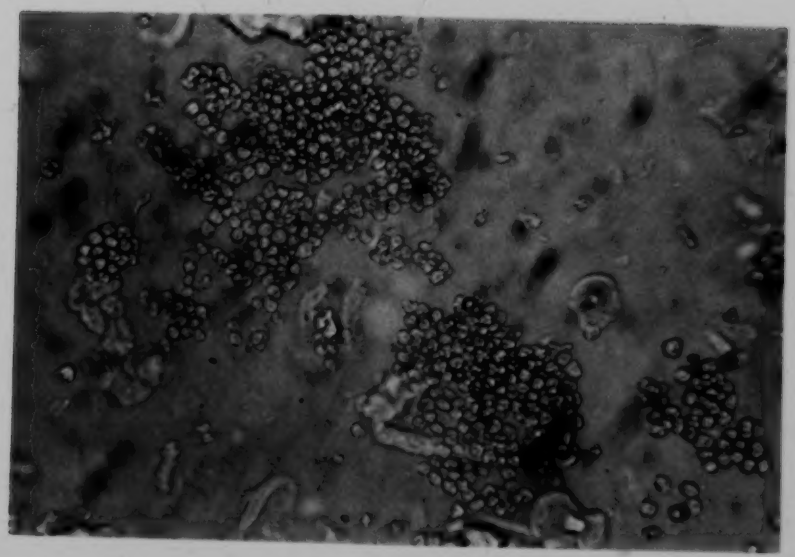


PLATE 5
HL-60 colonies after gamma IFN treatment (x10).

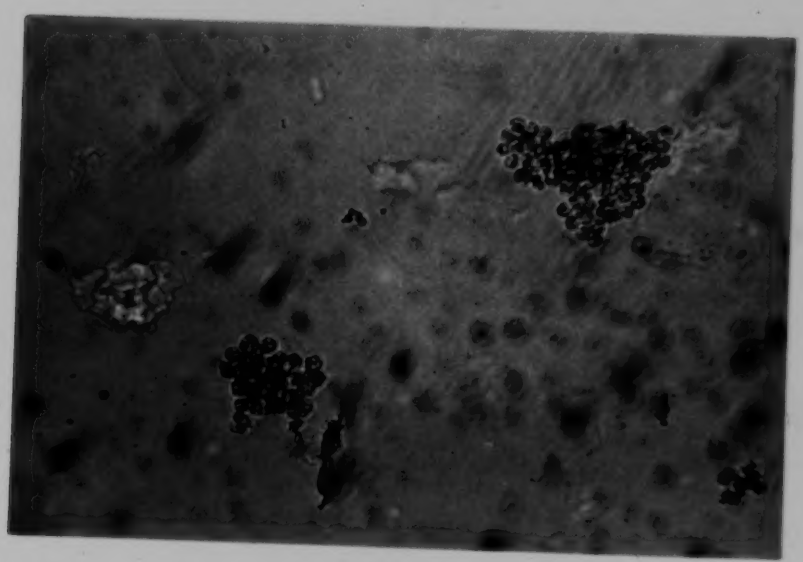


PLATE 6
KG-1a control colonies (x10).

PLATE 7
KG-1a colonies after treatment with gamma IFN (x10).

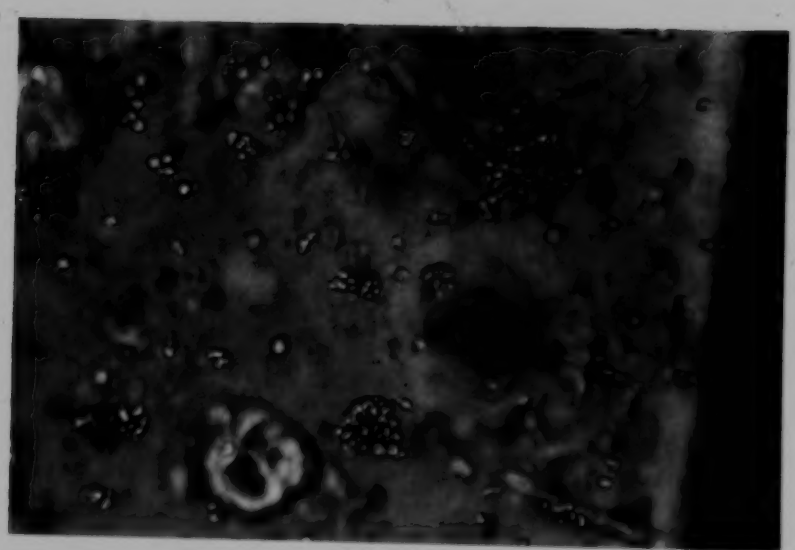
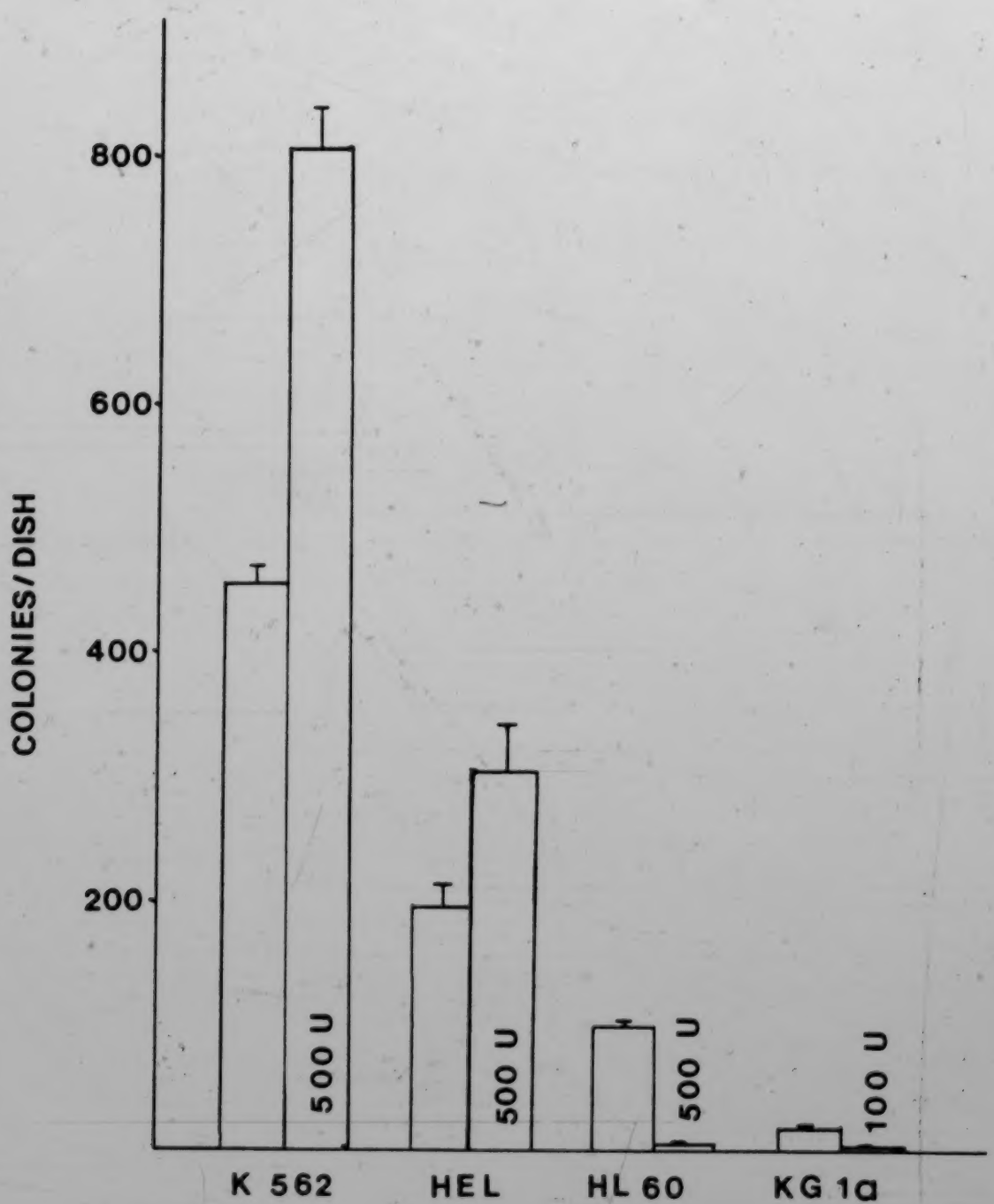


Fig.9
Effect of gamma IFN on CFUs.



also able to suppress colony formation by the myeloid cell line ML3 but not ML1 provided by Dr Mannoni (U of A) and Dr Minowada (RPMI, Buffalo, NY) respectively.

Recombinant alpha and beta interferons did not cause the same effect on HL-60 cells. 100 U/ml of alpha IFN did not inhibit colony formation while 500 U/ml had a significant suppressive effect. On the other hand, both 100 and 500 U/ml of beta IFN increased colony formation by two and threefold respectively (Fig. 10).

Performing the same experiments with the ML1 and ML3 cell lines we obtained no effect with alpha IFN and increase with beta.

Gamma IFN was unable to suppress colony formation by the two erythroid lines, HEL and K 562 (Fig.9). Dose response experiments on K 562 showed that the colony formation increases proportionally to the dose of gamma IFN (Fig. 11). No such an effect was observed with alpha and beta IFNs since 100, 500 and 1,000 U/ml of those agents did not significantly affect colony formation. The HEL line has a different behavior compared to K 562 since 500 U/ml of all IFNs gave a significant increase of CFUs (Fig. 12). It is clear that those chemically close molecules follow different

Fig.10
Effect of IFNs on HL-60.

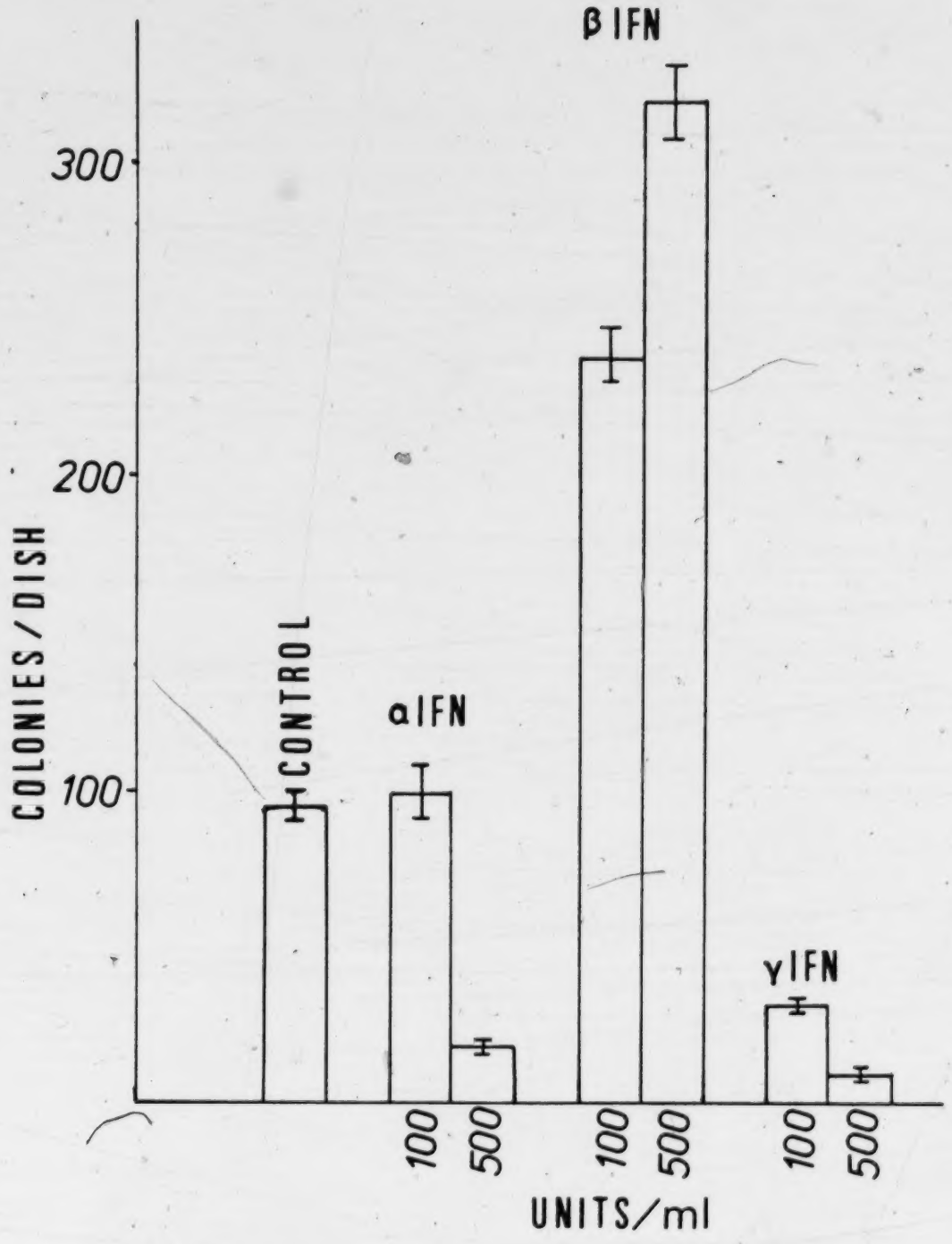


Fig.11
Dose effect of gamma IFN on K 562.

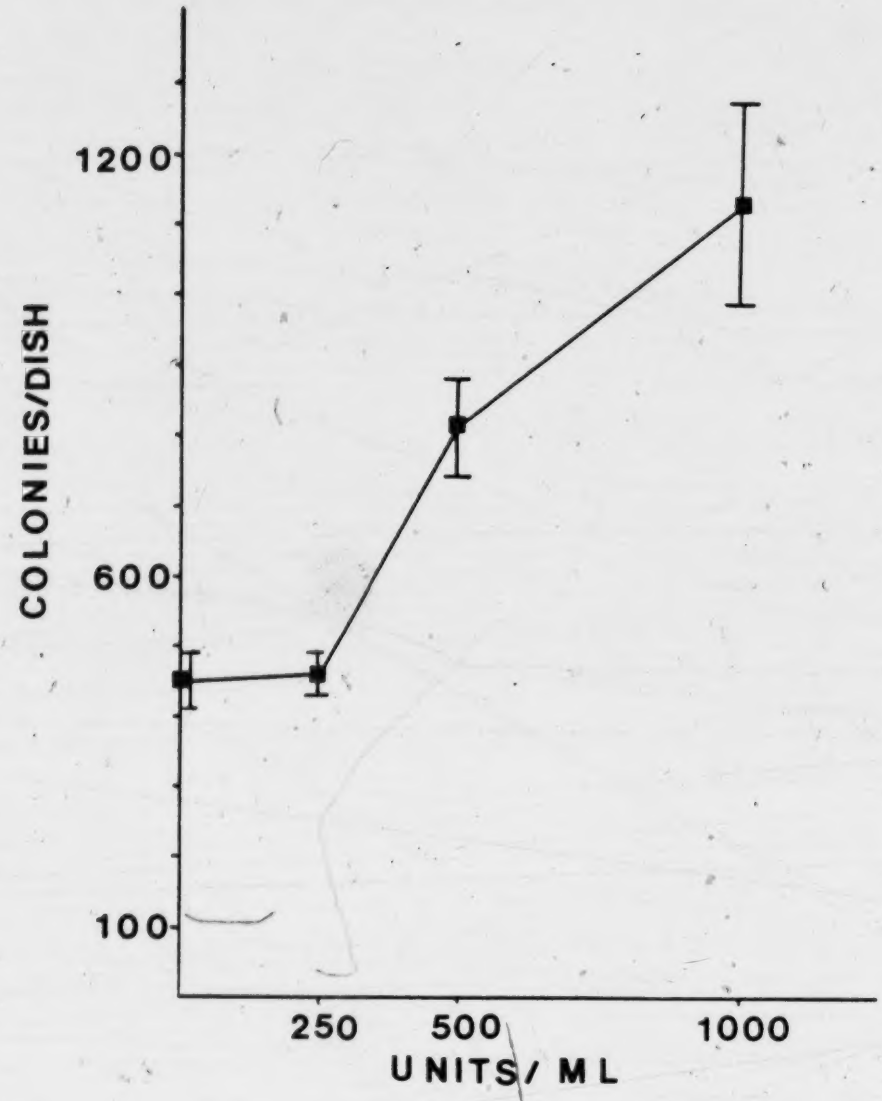
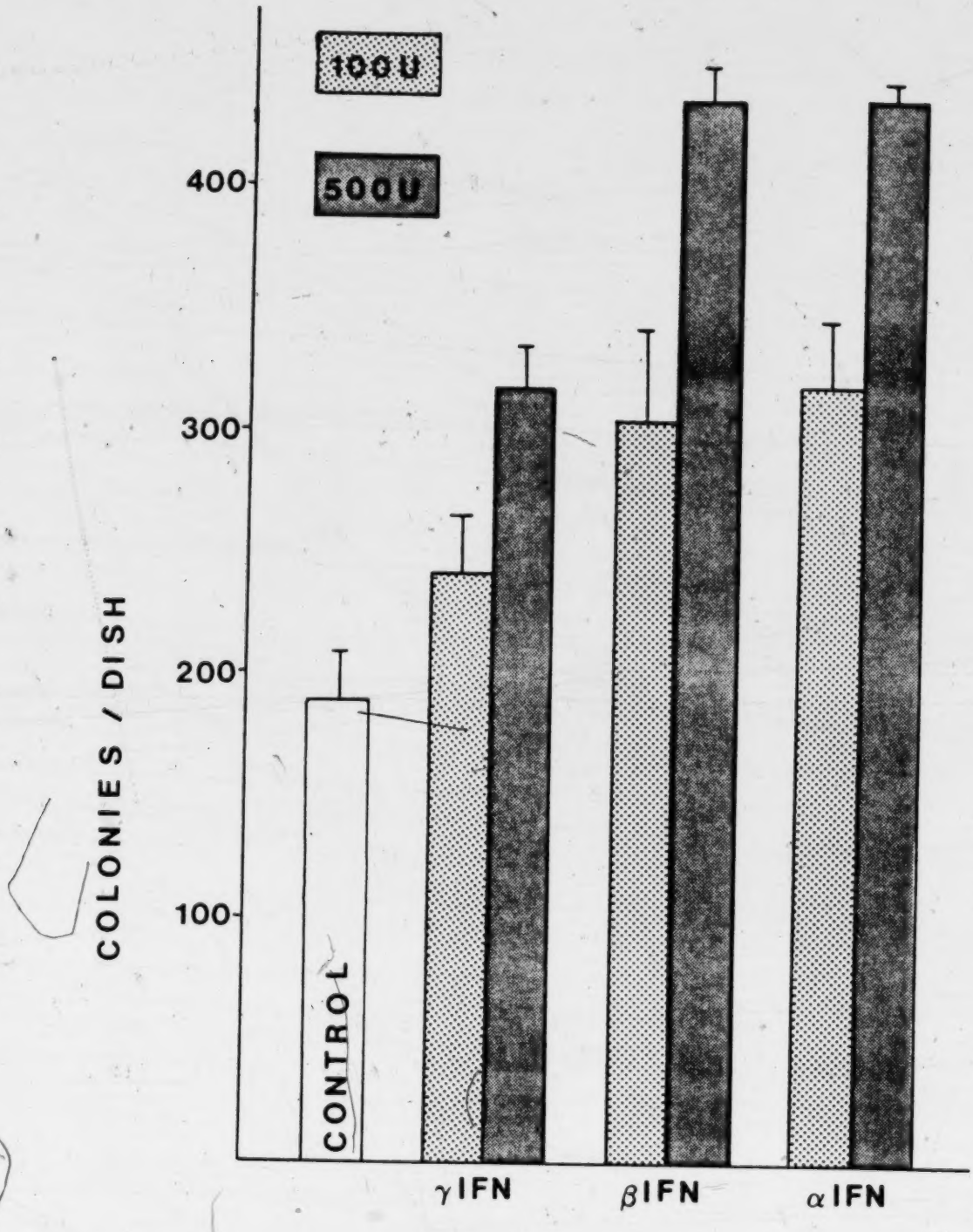


Fig.12
Effect of IFNs on HEL.



regulatory mechanisms according to the type of cells they are acting on.

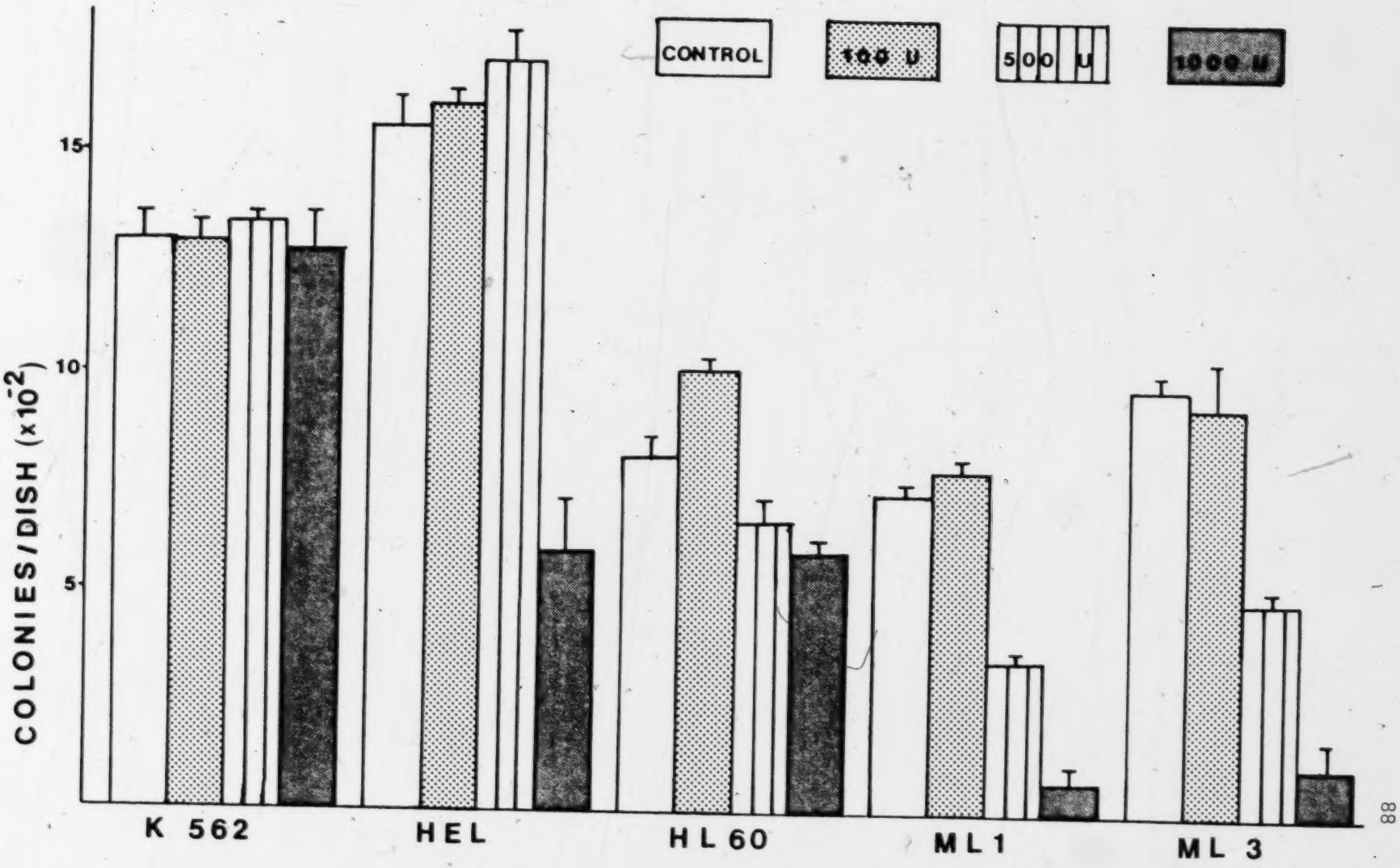
We then studied the effect of IL-2 on the differentiation process (Fig. 13). Since HL-60, which is the main cell line under investigation, expresses the T4 antigen we thought that this interleukin could have an important role on the differentiation of the line. We found that the number of colonies formed by the HL-60 cell line was not affected significantly by IL-2, while colony formation by the ML1 and ML3 lines was significantly suppressed at doses above 500 U/ml.

The two erythroid cell lines were not affected by different doses of IL-2, and only 1,000 U/ml decreased significantly the colony number of the HEL.

It has been shown that IL-2 stimulates the production of gamma IFN in normal peripheral blood mononuclear cells. This observation could be relevant to the suppression obtained by ML1 and ML3 lines, but it does not seem to be the case for HL-60.

One of the factors that regulate IL-2 and IFN production is PGE_2. This agent inhibited colony formation of the HL-60 line when used at a concentration of 10^{-7} M. The same agent inhibited the colony growth of the HEL line while it had no effect on

Fig. 13
Effect of IL-2 on different cell lines.



K 562 (Fig. 14). These observations confirm PGE_2's regulatory effect on erythropoiesis and support the theory that HEL has an inherent monocyte-like character (102).

Another finding was the action of hydrocortisone (10^{-6}M). It is known that this agent promotes hemopoiesis. However, in these experiments, we observed a highly suppressive effect which cannot be explained.

The effect of a number of conditioned media was also tested. Only the PHA-LCM medium decreased colony formation by HL-60, and this is probably due to the high amounts of gamma IFN it contained. This possibility is supported by the fact that PHA alone (0.5%) produced a two-fold stimulation. The Mo T supernatant had no effect while HPCM (Plates 8, 9), Mia PaCa and 5637 increased the number of colonies with the greatest effect being seen with 5637 (Fig. 15).

The effect of various supernatants (from bone marrow-Dexter type, and others elaborated from the contact between bone marrow and HL-60 or KG-1a) on CFUs was negative.

As it has been mentioned already, HL-60 expresses the T4 but not the T8 antigen which indicates the lineage infidelity concept (26,41,112) exhibited by

Fig.14
Effect of PGE_2 on cell lines.

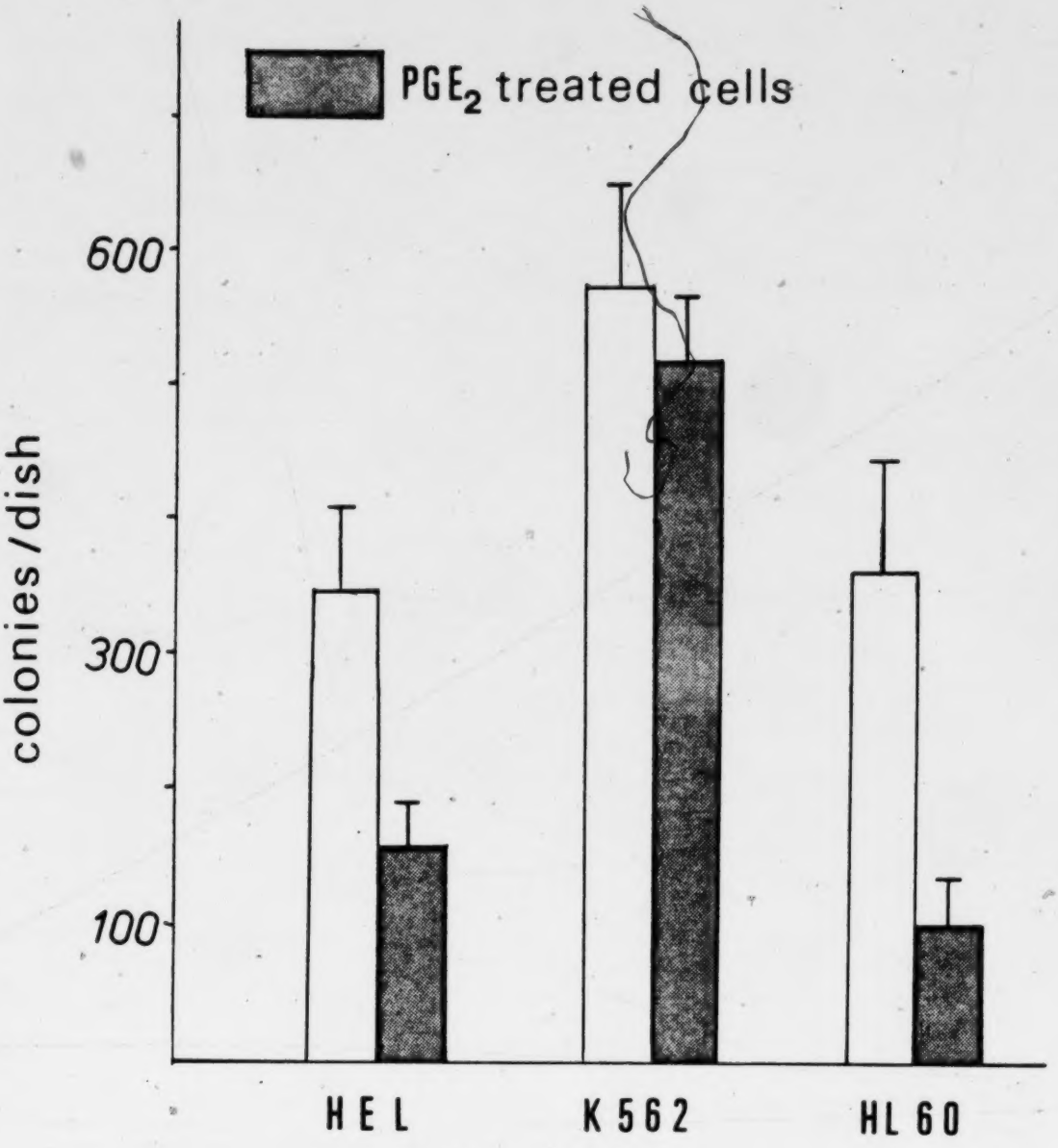


PLATE 8
HL-60 colonies after treatment with HPCM (x10).

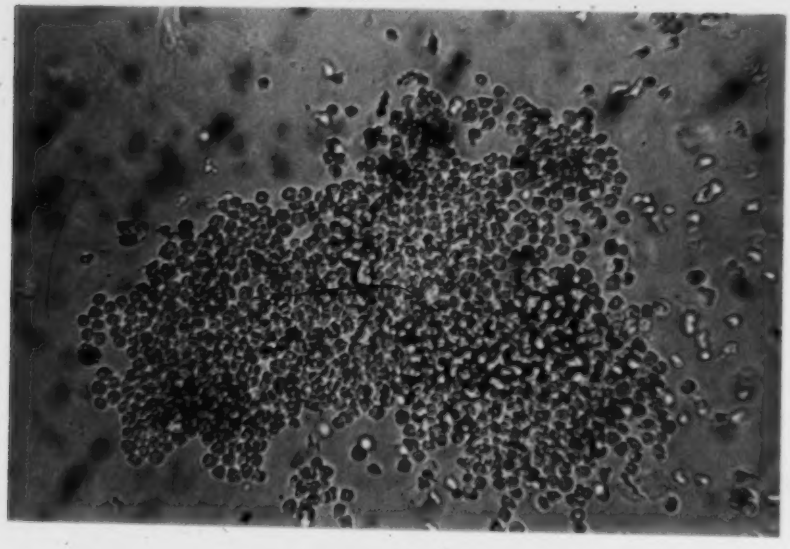


PLATE 9
KG-1a colonies after treatment with HPCM (x10).

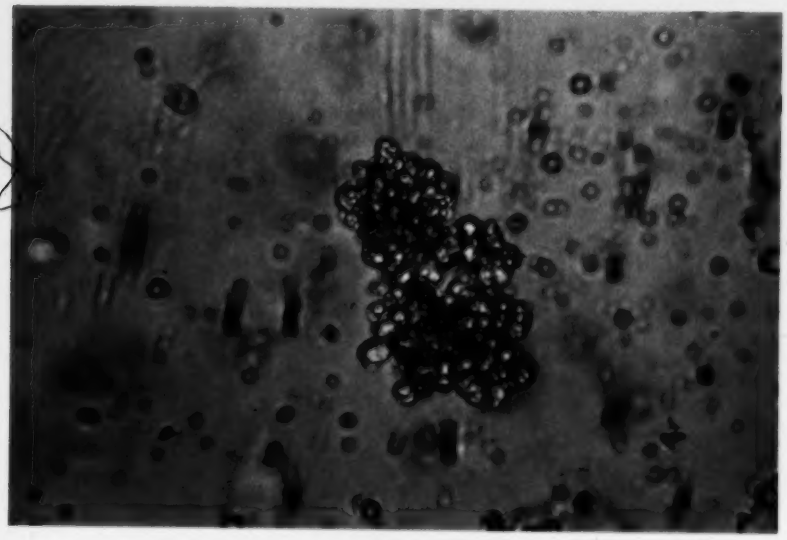
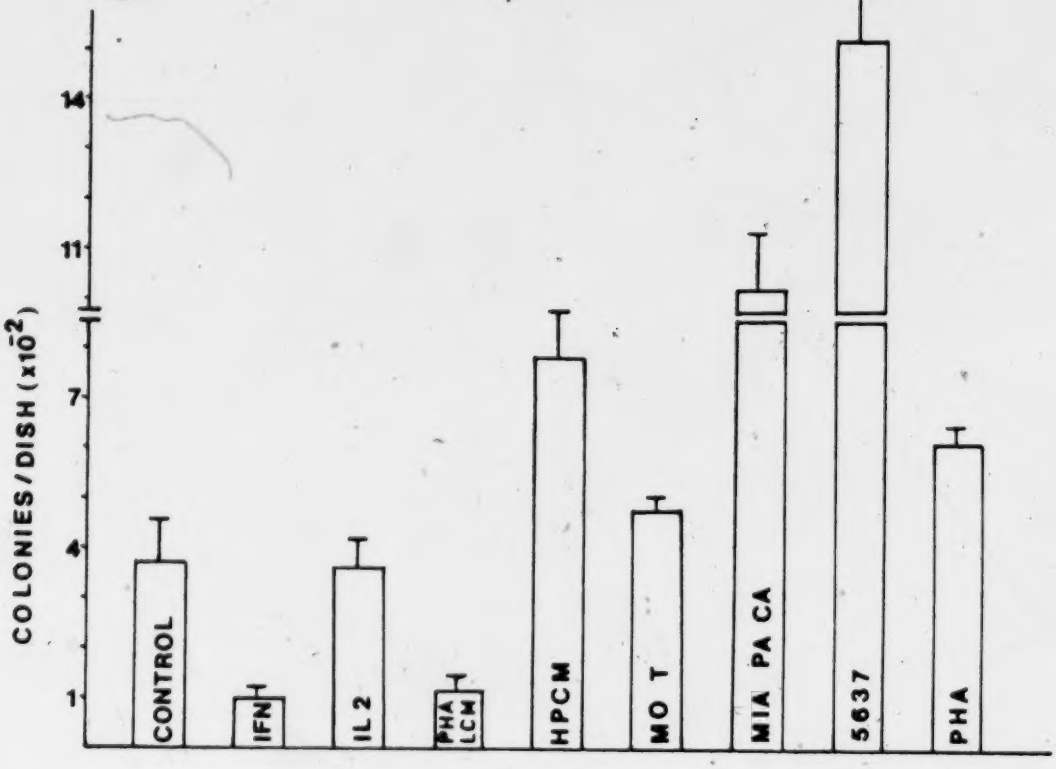


Fig.15
Control responses of HL-60 to various factors.



this cell line. We thought that the presence of the expressed antigen could have an important role on differentiation. We therefore plated the line with the T4 or T8 MoAbs and colony formation was assessed on day 12 of culture.

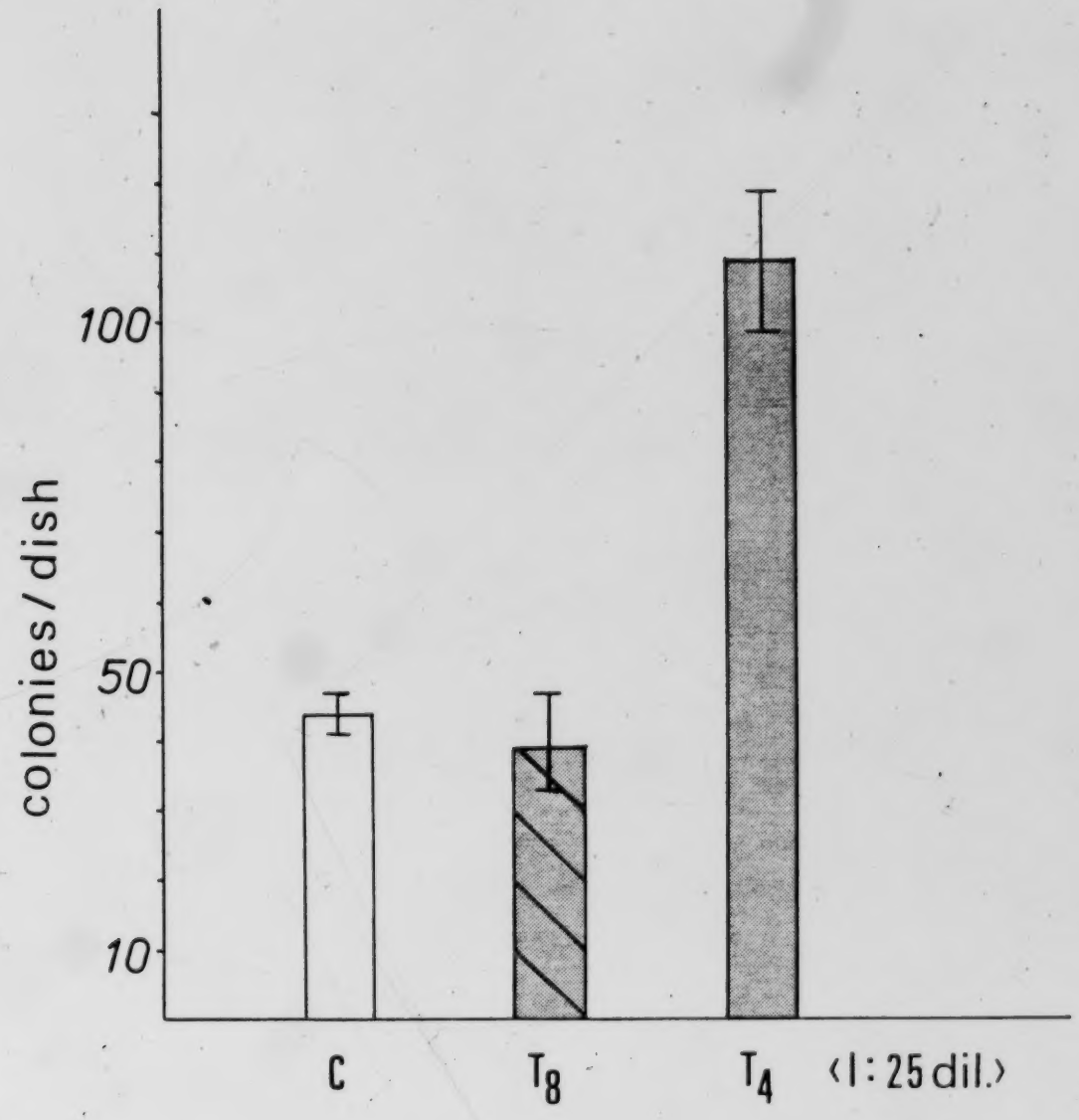
The T4 gave a threefold increase of colonies while the MoAb for the non-expressed T8 antigen had no effect (Fig. 16).

So far the results have shown that gamma IFN might be actively involved in differentiation. This view is also supported by observations made after long term incubation of HL-60 cells with gamma IFN.

As it has been reported for the mouse system (88), alpha and beta IFNs can modulate the behavior of cells towards different CSFs. A similar effect was obtained in our system by the addition of gamma IFN. In that series of experiments, we have been following the effect of gamma IFN for the period of four weeks. Every two to three days, cells were plated on methylcellulose and tested for their ability to form colonies.

During the first three weeks 100 U of gamma IFN per ml were added every two days to the cultures. This treatment caused a significant suppression of colony formation. In order to reverse the suppressive effect

Fig.16
Effect of T4 and T8 MoAbs on HL-60.



of this recombinant agent, we plated the same treated cells with a number of factors. All conditioned media tested (except PHA-LCM) were able to rescue the inhibition obtained. As suppression by IFN became more profound, 5637 was better able to counteract the suppressive effect, followed by an activity shared to a lesser degree by HPCM and Mo T. The Mia PaCa conditioned medium gave a stable positive response during the three weeks (Fig. 17). An extra addition of gamma IFN as well as PHA-LCM which was known to contain large amounts of IFN, caused a greater suppression. The fact that every CM used contains a battery of other components cannot give us any information about the mode of action of IFN. Trying to localize this effect, IL-2 was added to the treated cells. Although this lymphokine had absolutely no effect on the colony formation by untreated HL-60 cells, it could rescue the suppression significantly. However, the effect of IL-2 could be reversed when the gamma IFN treated cells were co-incubated with a cocktail of anti-Tac (IL-2 receptor) MoAb.

Some relative values of the potentiality of the CSFs during that period is given in Fig. 18 where the index of colonies (number of colonies divided by

Fig. 17
Effect of factors on HL-60 during long term treatment with gamma interferon.

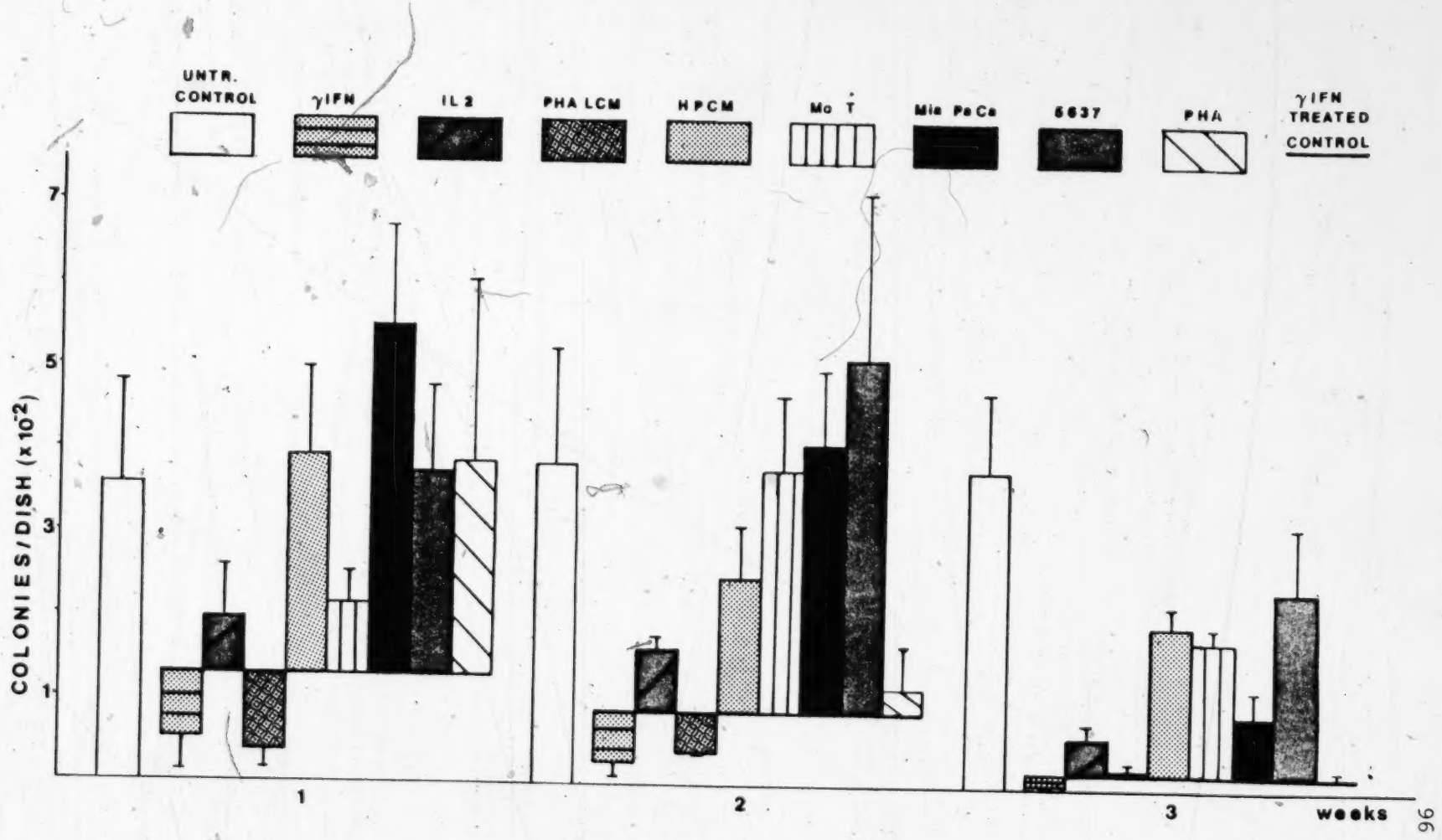
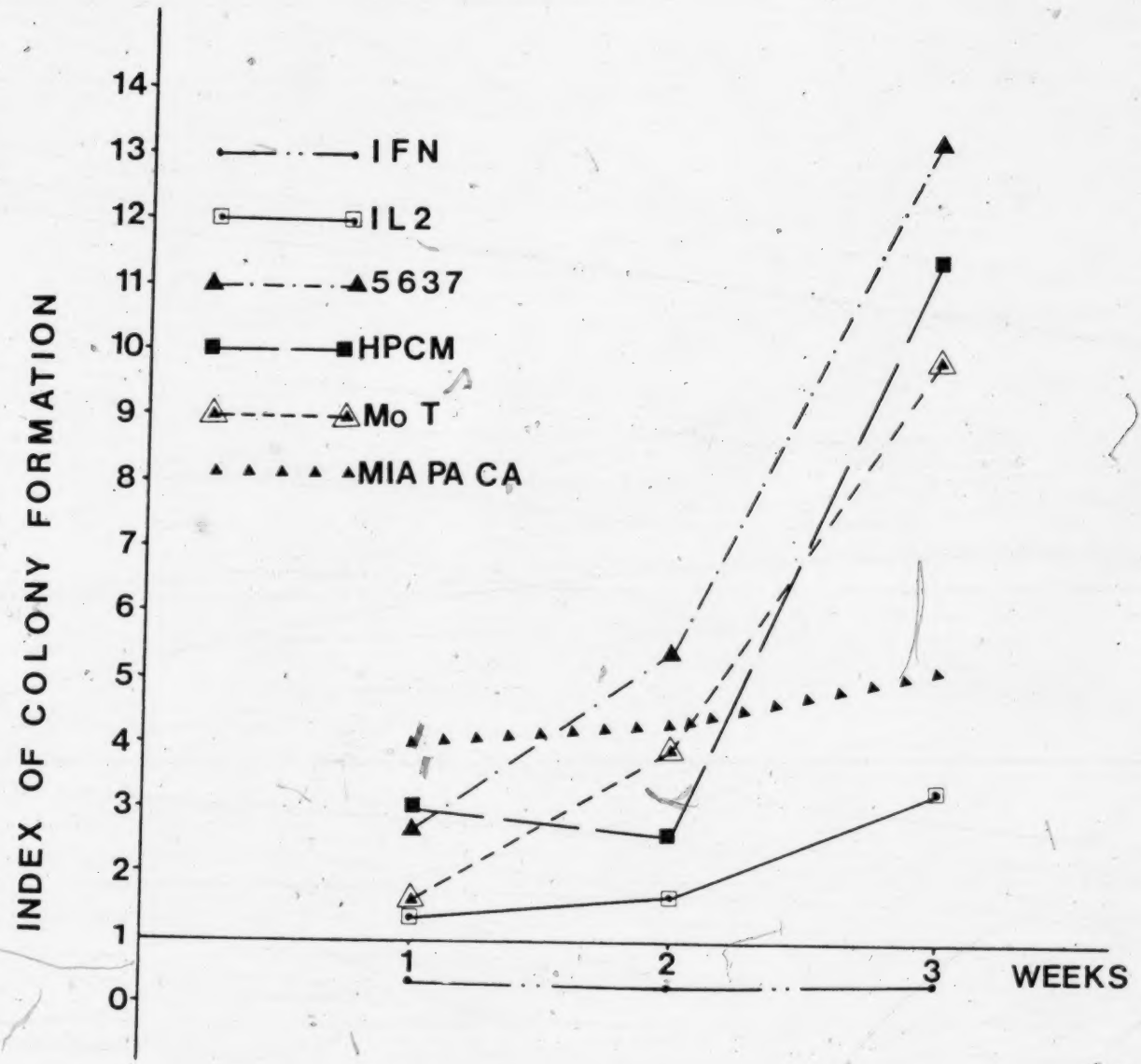


Fig.18
Time related factor effect.

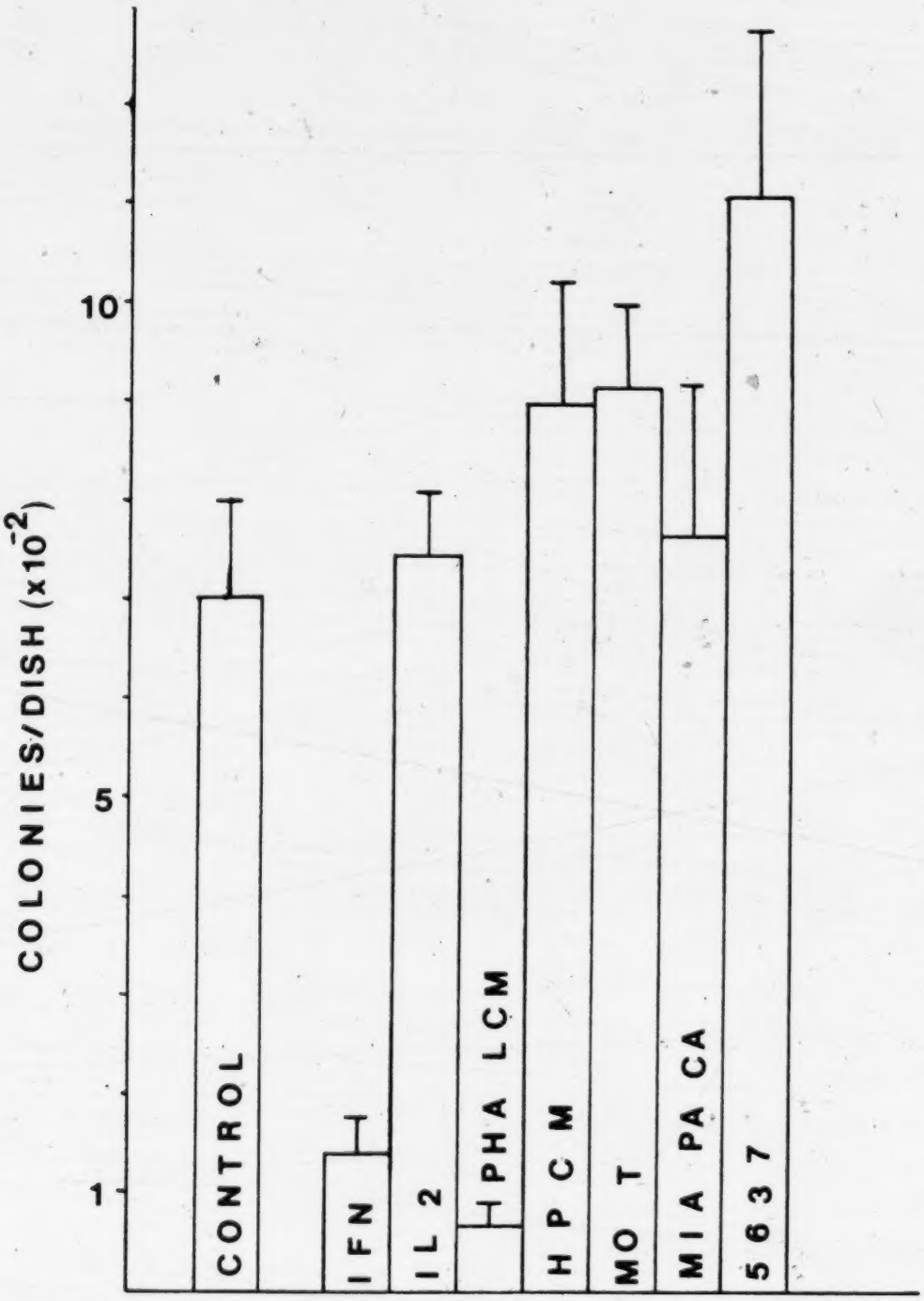


treated control colonies) is plotted against time. These numbers are compared to the gamma IFN treated control and are not absolute. The diagram represents the ability of these factors to rescue the cells from the gamma IFN treatment.

We have mentioned previously that gamma IFN treatment of HL-60 cells induces the appearence of two peaks corresponding to different class I populations. This observation made us think that this treatment reveals the presence of two differently differentiating populations. Therefore, a couple of subclones were produced by picking colonies and putting them in culture. They were named (Gallo-IFN)^1 and (Gallo-IFN)^2. The first one was derived from HL-60 cells that were treated with 100 units of gamma IFN only at the time of plating. The second one was derived from a three week long term treated cells, that were also plated with the same amount of IFN. After puting those cells in culture, we tested their ability to form colonies, and the expression of surface markers was characterized.

The (Gallo-IFN)^1 clone did not show resistance to IFN while the effect of all factors tested was comparable with those obtained during the third week (Fig. 19). The second clone, (Gallo-IFN)^2, showed

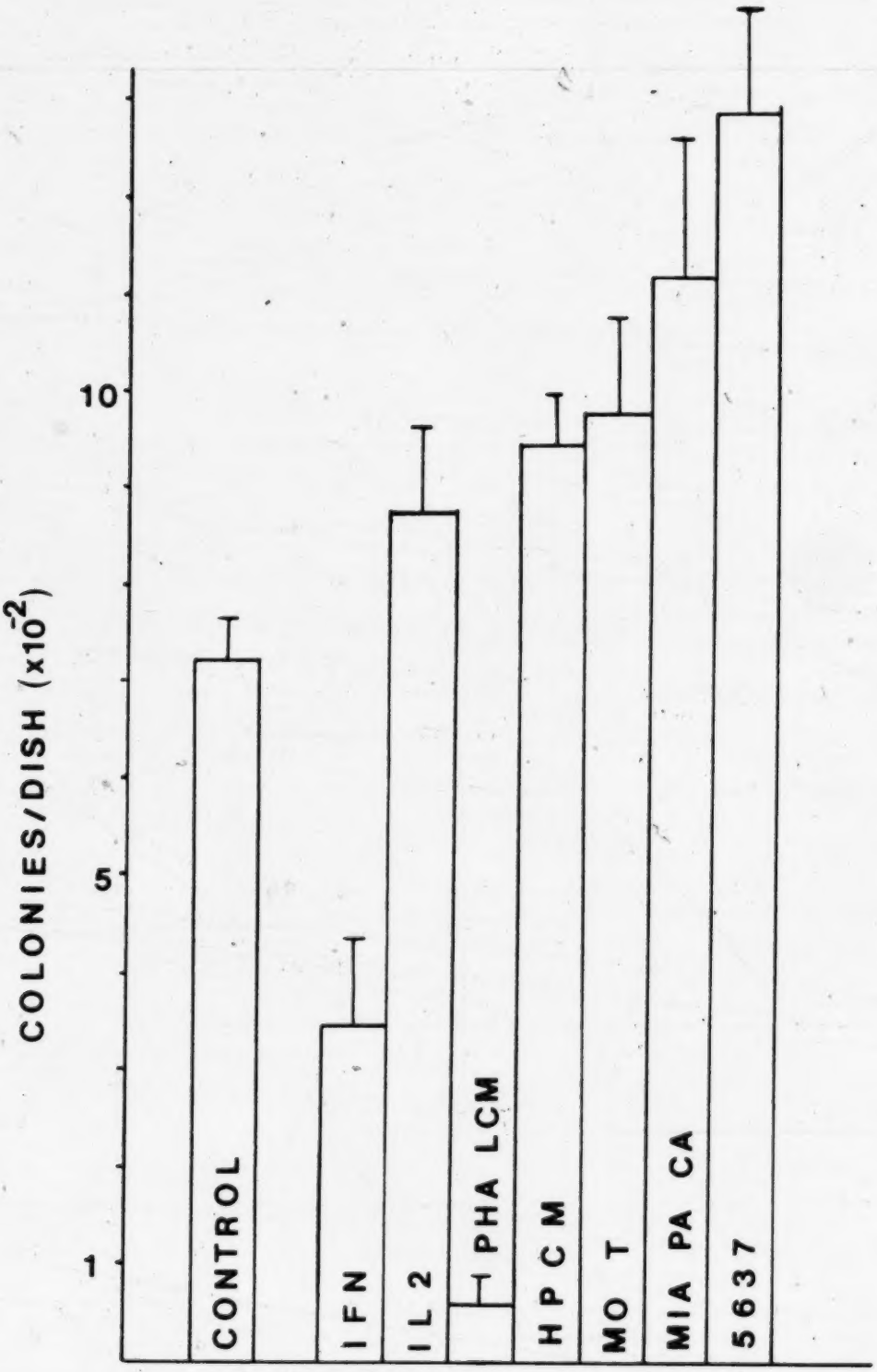
Fig.19
Colony formation capability of (Gallo-IFN)[1].



about 50% of resistance to IFN which was added at the time of plating. The best response was obtained from the 5637 CM and Mia PaCa while HPCM, Mo T and IL-2 gave similar responses, which were slightly higher than the treated control level (Fig. 20). Colony formation was greatly suppressed with PHA-LCM in both cases.

Both clones kept their original surface marker expression for class I and II antigens as well as for myelomonocytic markers. The only difference was the disappearence of T4 and 86H1 markers.

Fig.20
Colony formation capability of (Gallo-IFN)^2.



D. C-MYC EXPRESSION

Untreated and treated HL-60 Gallo cells were lysed and their cytoplasmic mRNA extracted and hybridized with a specific cDNA probe to assess c-myc expression. It is known that HL-60 cells constitutively express this cellular oncogene at very early stages of differentiation. Treatment of cells with alpha IFN does not decrease the c-myc transcript (16,29). In our system, treatment with gamma IFN resulted in an increase of c-myc expression that was especially evident after the second week and which was stable thereafter.

During the treatment, gamma IFN had always a suppressive effect on proliferation as assessed by ^{3}H thymidine incorporation experiments.

CHAPTER IV

DISCUSSION

It is widely accepted that in normal tissues, differentiation is inversely related to proliferation so that at a specific stage of maturation the cell loses its ability to proliferate. In the disease state, the cell maintains its ability to proliferate even after reaching a mature stage of differentiation. In fact, differentiation is a complex, highly regulated process based on selective activation and repression of genetic material. It appears to be a cytoplasmic event with no permanent changes to the cells genome (28). A violation of this concept is observed in the differentiation of B lymphocytes, where the generation of diversity in immunoglobulin specificity involves rearrangement of immunoglobulin gene segments (52). Cell differentiation is generally regarded as unidirectional and irreversible which ultimately leads to cell death.

In normal hemopoiesis the cells receive various stimuli and progress through stages according to the needs of the organism. Those stimuli are mainly due to

cell contact and to a great regulatory involvement of factors.

The use of cell lines as a relatively homogeneous population of cells provides an analytical tool for studying hemopoiesis and hemopoietic malignancies. They provide model-systems for investigating vital processes like cell differentiation and cell proliferation as well as providing insights into the cellular events linked to leukemia. By using cell lines, these studies can be performed under controlled experimental conditions and they also minimize the demand for "donor-type" cells.

In this work, we have studied the influence of various factors on proliferation, expression of surface antigens, differentiation and cellular oncogene expression of HL-60 while other myeloid and erythroid cell lines were used for comparative perpuses.

Gamma IFN has been studied for its inductive and modulatory efficiency. This factor is the most studied of the IFNs since its uses and potentialities have been well described. Its major known effect is on the expression of MHC antigens. In 1973, Lindahl et al. reported enhancement of class I antigens on mouse L

1210 cells after appropriate gamma IFN treatment (67). Since then, studies have shown that IFN's ability to increase surface antigen expression is not restricted to MHC antigens (107). Gamma IFN appears to be both modulator,able to increase the level of a MHC-gene product already synthesized and expressed (Fig.8), and inducer, able to induce the "de novo" activation of at least two classes of MHC genes. Its mode of action remains speculative. However, many models have been proposed that are based on its known ability to stimulate the transcription of the corresponding MHC's mRNA. It has been found that the enhancement of a cell surface antigen by IFN is always lower than the enhancement of the corresponding mRNA (108,45), but no explanation for this observation has been given.

In this work we confirm the inducer character of gamma IFN. This agent induces the appearence of class II antigens and increases the expression of class I. As assessed by flow cytometry analysis, a second population of class I positive cells appears after an appropriate gamma IFN treatment (Fig.8).

IFNs have also been studied for their modulator effect on progenitor cells. Their effect depends on the origin of the progenitor as well as on their degree of

maturation.

Alpha IFN at high doses inhibits myeloid colony formation whereas beta IFN is totally stimulatory. Both factors have no effect on erythroid lines.

It has been reported that these IFNs compete for the same receptor on the cell surface (13). Yet, no conclusion can be drawn since those agents have been less extensively studied.

Gamma IFN is known to inhibit CFU-GM of normal bone marrow. Initially it was believed that this effect was mediated through the T cells that were producing inhibitory substances responsible for the down regulation of colony formation (105). We have found in our laboratory that T cell depleted bone marrow still undergoes the same effect (P. Mannoni et al., manuscript in preparation). It is likely that gamma IFN can modulate surface receptors or/and act on the gene level so that other regulatory mechanisms involved supress colony counts. The same seems to be true for HL-60 which is a T cell independent cell line and exhibits the same suppressive pattern.

In order to modify the inhibitory effect of gamma IFN on the HL-60 cell line, we co-cultured long term

gamma IFN treated cells with different conditioned media and IL-2. All tested CSFs could reverse this effect but no information on the mechanic action of gamma IFN can be given since these results were not confirmed with recombinant factors. In addition, IL-2, a human recombinant lymphokine, could also rescue that suppressive effect. It has recently been reported (Dr F. Ruscetti, prersonal communication) that HL-60 bears receptors for IL-2. Thus, we speculate a feedback mechanism between these two agents regulating the differentiation process.

Similar results were also obtained with the ML3 cell line which may follow the same regulatory pathway.

Another important finding concerning gamma IFN is that HL-60 treated cells show an increase of the c-myc oncogene expression. That augmentation occurs at the second week of treatment and stays stable thereafter. Again, the mode of action of IFN seems to be similar as above since oncogene expression usually increases during cell activation (16).

Although this factor seems to have an important role in cellular differentiation it does not have any effect on proliferation.

In summary, we see that gamma IFN acts in a

synergistic way inducing the HL-60 cells to respond to IL-2 and CSFs. It also increases the c-myc expression by acting probably on the gene level. Our hypothesis is that c-myc triggers in turn other gene(s) that regulate surface receptors making the cells responsive to various stimuli.

The fact that HL-60 expresses the T4 antigen, associated with helper T cells, promted us to test whether IL-2, a T cell activating factor had an effect on this cell line. Its effect on proliferation, as assessed by ^{3}H thymidine uptake, is negative, and doses up to 500 U/ml do not affect colony formation of both HL-60 and erythroid cell lines. For other myeloid lines (ML 1, ML 3) doses higher than 100 U/ml do suppress colony formation. This difference between HL-60 and ML 1/ML 3 can be attributed to the different nature of the lines. The ML lines are derived from patients with acute myeloblastic leukemia and, as depicted into Fig.3, they are evolutionary, one step behind the HL-60 line.

Colony-Stimulating Activity (CSA) is the term given to a group of polypeptide molecules capable of stimulating granulocyte and monocyte colony formation

in semisolid culture (85). This growth-promoting hormone is believed to be a physiologic granulopoietin and has been isolated from various murine and human tissues (39). Most human sources of CSA consist of growth medium conditioned by cultured cells. Significant amounts of those are released from placental cells, monocytes, T cells, endothelial cells and cell lines of pancreatic and lung neoplasms. The action of CSA appears to be specific. It has been separated into a group of related glycoproteins called Colony-Stimulating Factors (CSF) and these factors fully account for the ability of CSA to induce colony formation (37,56). The release of CSA appears to be controlled by humoral interactions between cells and by cell to cell contact (37).

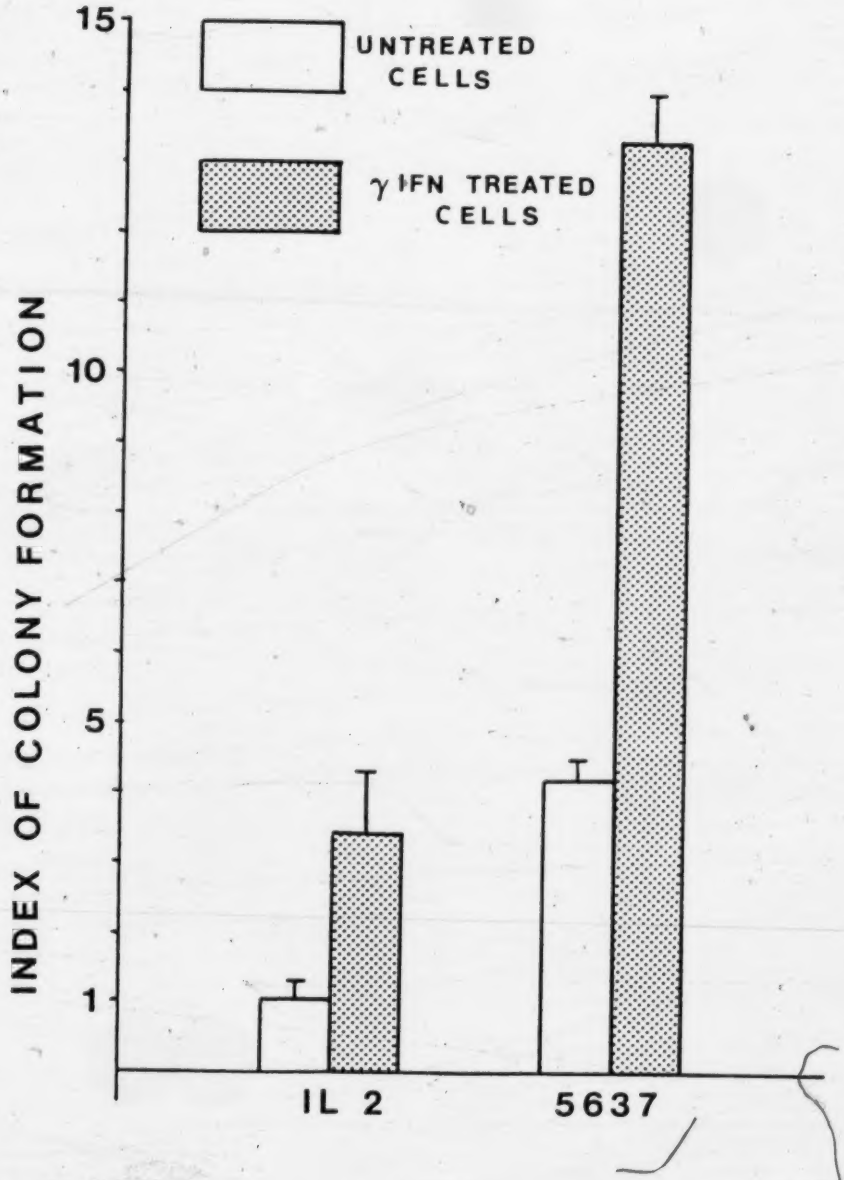
Purified preparations give the most potent results but partially or non-purified factors can have similar effects. Purification can lead to the resolution of the CSFs into further components. For example, HPCM "contains" two CSFs that have been separated on the basis of differences in hydrophobicity: CSF-alpha and CSF-beta, each fraction stimulating different cell-types (97,98). These two CSFs have been also reported in the 5637 CM (96).

In this study, their effect on proliferation was negative with the exception of HPCM, which when assessed on day four of culture stimulated the proliferation. In contrast, interesting observations were made on the expression of surface markers where all of the CM used could induce the 82H3 monocytic marker.

In addition, from all CM used, the 5637 and HPCM could increase the expression of class I antigens the way gamma IFN did. Despite the fact that these media are IFN free, two different peaks were obtained denoting two populations having different responses to class I antigens. Comparing their effect with gamma IFN we can suggest that the former mimics CSF action and could be classified as a hemopoietin.

The effect of CSFs on differentiation as assessed by colony assays,was stimulatory with the exception of PHA-LCM which contains gamma IFN. Mia PaCa and 5637 gave the best results by stimulating colony formation when the HL-60 cells were treated with gamma IFN for a prolonged period of time. Here again, gamma IFN seems to modify the responsiveness of the cells towards different CSFs as is the case also with IL-2. This is demonstrated in Fig. 21 where the index of colony

Fig. 21
Demonstration of the regulatory effect of gamma IFN.



formation is plotted for untreated and gamma IFN treated cells. It can be said that gamma IFN induces the cells to respond to various factors.

It has been clearly shown so far that proliferation and differentiation are different processes. Treatments that promoted or suppressed differentiation were unable to modify cell division. In an attempt to study proliferation we used Human Bone Marrow Feeder Layer (HBMFL) which, in this work, provides a model-system for studying cell growth. The results indicate that a species-specific mechanism regulates and/or supports a proliferative response based probably on a cell to cell interaction pattern. This inference is drawn from the observations that bone marrow supernatants are ineffective on the one hand, and on the other hand, interruption of the two populations by a thin layer of methylcellulose prevents proliferation. The exact identity of the adherent cell(s) that provide the stimulus is still unknown. It is likely that a certain type of cell(s) at a certain stage of maturation is responsible for the resulting increase in proliferation. This view is supported by the fact that the "age" of the feeder is important

since a layer older than four months cannot support the growth of the seeded cells. This "failure" coincides with the appearence of class II antigenic determinants (data not shown).

This increased proliferation pattern can be also attributed to the fact that bone marrow or its hemopoietic microenvironment can promote leukemia since malignancies start from the marrow level. Perhaps this is the in vitro analogous system, since cells escape surveillance within the marrow environment.

In an attempt to identify the kind of interaction and nature of the cell(s) involved in this system, we treated the feeder layer with a number of MoAbs. Unfortunately, we cannot formulate any conclusions because the percentage of suppression was only 46% (mean value). Additionally, the use of MoAbs in the present case is too vague. We should first try to identify the type of cell(s) that promote proliferation by producing feeders with different cell lines that are quite homogeneous in population and then try to examine the kind of interaction involved using more spesific antibodies.

The significance of the inability of mouse feeders to support cell growth is still not clear. It could be

the result of a species-specific interaction or it may be due to a number of inhibitors that the mouse cells may elaborate. These questions can be further analyzed using different sources of cells for the layer and the overlayer, including combinations of mouse and human cells.

The human-human system appears to be a powerful tool for monitoring normal and leukemic cell proliferation. Bone marrow supernatants do not promote any further cell differentiation as assessed by colony assays or surface marker expression on leukemic cells as tested by indirect immunofluorescence.

SUMMARY AND CONCLUSIONS: In this work we have shown the effect of gamma-IFN, IL-2 and CSFs on myeloid cell growth. Based on the effect of gamma-IFN on colony formation and on its role on proliferation we can hypothesize the possible role of this agent: Gamma-IFN seems to favor differentiation since a progenitor Po can mature to a progenitor Pl. At this point gamma-IFN inhibits proliferation and Pl is unable to give a colony of Pl cells. This inhibitory action can be removed by the addition of IL-2 and CSFs which, as we have seen, can "rescue" the response of gamma-IFN treated cells by giving higher numbers of colonies. The role of IFN as a differentiation agent can be further investigated by means of molecular biology techniques such as, isolation of a specific chromosome (i.e. #6, HLA) with an appropriate cDNA probe and then by studying the lenght of treated and untreated fragments. Another way is to use electron microscopy, trying to localize splicing(s) that will confirm the differentiation process.

A battery of factors, with the exception of HPCM, was not able to induce proliferation on HL-60. This was expected, since the cell line under study could proliferate by itself in the growth medium without the

need of exogenous factors. The fact that human bone marrow feeder layers were able to increase the proliferative response of HL-60, even at very low concentrations of FCS, suggests that the layer system can act in synergy with cytoplasmic or nuclear factors that induce the cells to respond in the absence of CSFs or FCS. Therefore, the HL-60 line can be viwed as a model for studying proliferation. But to a certain extent, it can be also used as a model for differentiation using the colony assay, since: a) HL-60 responds to the same factors which induce differentiation in the normal bone marrow, b) it does not respond to all CSFs in the same way (this has to be confirmed by recombinant factors) and c) its responsiveness to factors can be modified by gamma IFN. If this can be also confirmed in the bone marrow, then, the simple model of HL-60 may indicate that hemopoiesis can be seen as a cascade of different events involving cell to cell interactions, presence of specific factors or/and specific receptors. A second, more complex, hypothesis can be formulated involving an intrarelationship between oncogenes like c-myc, and specific factors/receptors. In order to prove this, it is necessary to use a wide selection of cDNA probes for all oncogenes and work with recombinant CSFs.

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